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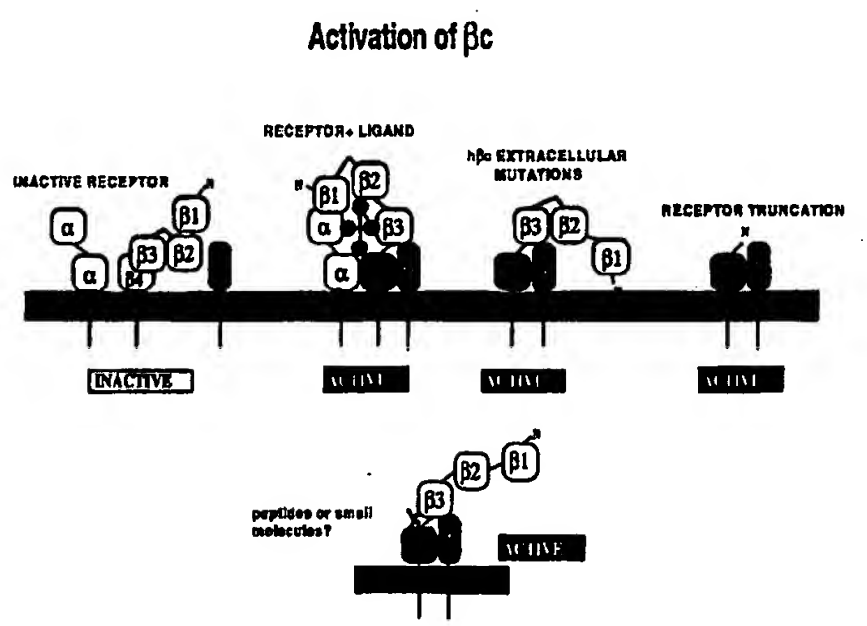

(57) Abstract

A therapeutic agent said agent being an agonist to a receptor of the cytokine receptor family such as the hβc or common β receptor of IL-3, IL-5 and GM-CSF. Each of the members of the cytokine receptor family having an extracellular receptor module (CRM) composed of two discrete folding domains (CRD). It has been found that a first of the two CRDs, in the case of hβc being β4, is a switching CRD so that conformational changes in the switching CRD lead to activation. A second of the CRDs, in the case of hβc being β3, being an inhibiting CRD. Under normal conditions the CRM is in an inactive state unless activated by ligand. The agonist property being a result of a change in the switching CRD from its inactive state to an active state leading to a signal being produced to effect said agonist property, and may be a result of blocking the inhibitory effect of the inhibiting CRD on the switching CRD or may be a result of inducing a change to the switching CRD by itself.

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AGONISTS OF HAEMOPOIETIC GROWTH FACTORS

FIELD OF THE INVENTION

This invention relates to agents that act as agonists to certain Haemopoietic Growth Factor receptors and members of the cytokine receptor family, the therapeutic use of such agonists, and methods of isolating such agonists.

BACKGROUND OF THE INVENTION

The present invention arises as a result of findings that explain the mechanism of action of the common β subunit of the receptor for the Haemopoietic Growth Factors GM-CSF, IL-3 and IL-5 (hereinafter called the hβc). It is to be understood, however, that this mechanism is unlikely to be limited to only this receptor because of the structural similarities with other receptor molecules triggered by Haemopoietic Growth Factors (HGFs) and other members of the cytokine receptor family.

INTRODUCTION

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) stimulate the proliferation, differentiation and functional activity of a wide variety of haemopoietic cells including neutrophils, eosinophils, monocytes and early progenitor cells (reviewed by Metcalf, 1986; Clark and Kamen, 1987). In addition, the capacity of GM-CSF and IL-3 to stimulate the proliferation of eosinophil progenitors is also shared by interleukin-5 (IL-5) (reviewed by Sanderson, 1992). This functional overlap, as well as the cross-competition for binding to receptors on the surface of human haemopoietic cells (Lopez *et al.*, 1991), has a clear correlate in the structure and composition of the receptors for these three CSFs.

The high-affinity receptors for human GM-CSF (hGMR), IL-3 (hIL-3R) and IL-5 (hIL-5R) are composed of ligand-specific α subunits (hGMRα, hIL-3Rα and hIL-5Rα) associated with a common β subunit (hβc). The α subunits bind their cognate factors with low affinity, whereas hβc alone does not detectably bind any of these factors but is required in association with the α subunits to confer high-affinity binding (Gearing *et al.*, 1989; Hayashida *et al.*, 1990; Kitamura *et al.*, 1991a; Tavernier *et al.*, 1991). Moreover, hβc is essential for signal transduction (Kitamura and Miyajima, 1992; Sakamaki *et al.*, 1992; Kitamura *et al.*, 1991b), and as a shared signal-transducing component, provides a molecular explanation for the overlapping biological characteristics of GM-CSF, IL-3 and IL-5.

The hβc receptor is a member of the cytokine receptor family which are active with a very large number of cytokines (reviewed in Miyajima *et al.*, 1992, which review is

incorporated herein by reference). Members of the cytokine receptor family are characterised by a 200 amino acid extracellular receptor module (CRM) composed of two discrete folding domains (CRDs), each of which contains seven β strands folded into anti-parallel β sandwiches, and bears a structural similarity to the immunoglobulin constant domains (Bazan, 1990; Kaczmarski and Mufti, 1991). This predicted structure has been confirmed for three members of the family; the growth hormone, prolactin and EPO receptors, by X-ray crystallographic studies (De Vos *et al*, 1992; Somers *et al, 1994*). In addition to this similarity in tertiary structure members of this superfamily share a number of conserved sequence elements; (1) four conserved cysteine residues located in the N-terminal domain, (2) a membrane proximal Trp-Ser-Xaa-Trp-Ser (where Xaa is any amino acid) motif, also known as the "WSXWS box", located in the C-terminal domain (Bazan, 1990, Cosman *et al*, 1990, Fukunaga *et al*, 1991) (3) a proline rich motif (PRM) which may be involved in signal transduction located in the membrane proximal region of the cytoplasmic domain of most receptors in this family (O'Neal and Lee, 1993; Benit *et al*, 1994).

hβc is a glycoprotein of 130kDa and has two repeats of the cytokine receptor motif described above (Hayashida *et al*, 1990) and therefore has four discrete folding domains (CRDs) each referred to as β1, β2, β3 and β4. Residues in the membrane proximal domain of hβc (β4) have been identified as critical for affinity conversion by hβc, presumably through an interaction with residues in the first helix of the growth factor (Woodcock *et al*, 1995). To date there is little information regarding the role of the first three domains of hβc in ligand binding.

Whilst there is some understanding of the intracellular pathways involved in signalling via hβc (Ihle *et al*, 1995), there is still an incomplete understanding of the stoichiometry of the active receptor complex and the precise role of the α and β subunits in signalling. At least two distinct regions of the intracellular domain of hβc are involved in the generation of separate intracellular responses (Sakamaki *et al.*, 1992; Sato *et al.*, 1993) which implies that hβc is associated with and utilises multiple effector molecules. The mechanism by which the effector molecules are activated is not clear. While it is accepted that signalling is mediated by a complex comprising the ligand and both the α and β subunits, neither the precise stoichiometry nor the role of each subunit in signalling is clear. On one hand, the cytoplasmic portion of the α subunit is required for signalling by the GM-CSF and IL-5 receptors (Sakamaki *et al.*, 1992; Takaki *et al.*, 1993; Polotskaya *et al.*, 1994). On the other hand, studies with chimaeric receptors suggest that dimerisation of the intracellular portion of the β subunit may be sufficient to initiate signal transduction (eg Sakamaki *et al.*, 1993; Takaki *et al.*, 1994).

A role for hβc oligomerisation is also suggested from hβc constitutive mutants. Hannemann *et al* (1995) recently isolated a truncated murine βc subunit from a spontaneous factor independent mutant of the promyelocytic cell line, D35. In this receptor the extracellular domain has been replaced by 34 amino acids encoded by intron 10 sequences. The authors postulate that the replacement of the normal extracellular sequences with the intron encoded segment facilitates homodimerisation perhaps through an extracellular cysteine bridge. An hβc mutation in which Glutamic acid is substituted for Valine 449 in the transmembrane domain confers factor independent growth in FDC-P1 and Ba/F3 cells (Jenkins *et al.*, 1995). This mutation is analogous to the activating mutation in the *c-neu* proto-oncogene (HER-2, *erb*-B2 receptor) which leads to receptor oligomerisation via a transmembrane mediated association (Weiner *et al.*, 1989; Sternberg and Gullick, 1989).

Activating mutations have also been characterised in other cytokine receptors. A constitutively active form of erythropoietin receptor (EPO-R) containing an arginine to cysteine substitution in the membrane proximal CRD (at position 129) has been described. This R129C form of EPO-R forms disulphide linked homodimers in the absence of EPO suggesting that wild type EPO-R is activated by ligand induced homodimerisation (Watowich *et al* 1992). The introduction of further cysteine residues into the EPO-R membrane proximal domain also leads to disulphide linked homodimers that are constitutively active (Watowich *et al.*, 1994). *v-mpl* is a murine oncogene encoding c-mpl (a similar market) a fusion protein and is transduced by the murine myeloproliferative leukaemia virus (MPLV). The *v-mpl* fusion was generated from the partially deleted and rearranged *env* gene fused with cellular sequences from the *c-mpl* proto-oncogene encoding the thrombopoietin (TPO) receptor (Kaushansky *et al.*, 1994;, Bartley *et al.*, 1994).

Whilst HGF have proved therapeutically useful, they are large proteins that have to be administered parenterally. The possibility that small molecules might replace these has been an attractive concept, but small molecules based on ligand are likely to be too complicated having to bind to the two (or more) receptor chains. Small molecules that sterically alter the receptor or induce an active conformation (in this case βc) therefore could provide a similar spectrum of activity as ligand and be orally available. This advance would make therapy easier and possibly cheaper. The fact that these molecules would need to interact with receptor extracellularly would potentially simplify their design in not needing cellular penetration for function.

By way of a shorthand notation the following three and one letter abbreviations for amino acid residues are used in the specification as defined in Table 1.

Where a specific amino acid residue is referred to by its position in the polypeptide of an protein, the amino acid abbreviation is used with the residue number given in superscript (i.e. Xaa^n)

TABLE 1

Amino Acid	Three-letter Abbreviation	One letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

SUMMARY OF THE INVENTION

A model of activation of hβc has been developed and in particular of the β4 domain which proposed a structure consisting of 7 β strands formed into two hinged sheets, sheet one comprising of strands E, B and A and sheet two comprising of strands D, C,

F and G. Sheets 1 and 2 interact especially via strands B and C. It is also proposed that an interaction exists between the β3 domain and the β4 domain that maintains hβc in an inactive state, unless stimulated. It is proposed that a conformational switch occurring in the β4 domain is an essential step in the activation process. Thus certain mutations that prevent interaction of β3 with β4 lead to constitutive activity, and certain mutants that disrupt the interaction between sheet 1 and sheet 2 of β4 also lead to constitutive expression. Some agents that specifically bind one of the two region, β3 or β4 may disrupt the inhibitory interactions and thus these agents will act as agonists.

It is expected that a similar mechanism is operational for other members of the cytokine receptor family. The overall structure of other members will be to have at least one extracellular receptor module (CRM) composed of two discrete folding domains (CRD), a first of the two CRDs will be a switching CRD and will be equivalent in function to β4 in hβc and a second of the CRDs is an inhibiting CRD which is equivalent in function to the β3 of hβc.

Thus in a first aspect the invention could be said to reside in a therapeutic agent said agent being an agonist to a receptor of the cytokine receptor family having an extracellular receptor module (CRM) composed of two discrete folding domains (CRD), a first of the two CRDs being a switching CRD and a second of the CRDs being an inhibiting CRD said CRM being in an inactive state unless activated by ligand, said agonist property being a result of a change in the switching CRD from its inactive state to an active state leading to a signal being produced to effect said agonist property.

The agonist property of the agent may be as a result of its interference with the interaction of the inhibiting CRD with the switching CRD.

Alternatively the agonist property of the agent may be as a result of its interference with the interaction between sheet 1 and sheet 2 of the switching CRD. The agent may specifically bind with a portion of the switching CRD including the B or the C strand of switching CRD, or a least more specifically the agent may interfere with the interaction between any one or more of isoleucine 374, leucine 356 or tryptophan 358 and an apposed amino acid in hβc or analogous amino acids in another members of the cytokine receptor family.

The therapeutic agent may be a peptide with an amino acid sequence present in the inhibiting CRD or the switching CRD. The agent may have an amino acid sequence present in the B strand or the C strand of switching CRD. The agent may include a

sequence including the membrane proximal WSXWS motif or the YXXRVRVR motif located in the switching CRD. Alternatively the agent may have an amino acid sequence present in either the B or the C strand of switching CRD and includes any one or more of isoleucine 374, leucine 356 or tryptophan 358 in hβc or analogous amino acids in another member of the cytokine receptor family.

The agent may have agonist properties to a member of the cytokine receptor family where the member is selected from any one of a group acting as a receptor for any one or more of the following cytokines GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-13, IL-14, IL-15, granulocyte colony stimulating factor (G-CSF), erythropoietin (EPO), thrombopoietin (TPO) and leptin, or the agent may be an agonist of any one of GM-CSF, IL-5 and IL-3.

The agent may be selected from any one of a number of classes of compounds comprising antibodies, fragments of antibodies, peptides, oligosaccharides, oligonucleotides, or other organic or inorganic compounds.

The agent may be capable of binding one or more of those parts of the inhibiting CRD or switching CRD that are conserved within the family of haemopoietic growth factor receptors.

The agent may be capable of binding a region of the switching CRD domain including at least a portion of the B strand or the C strand or at least the WSXWS motif or the YXXRVRVR motif.

The agent may be capable of binding a region of the switching CRD domain including isoleucine 374 or leucine 356 or tryptophan 358 in hβc or analogous amino acid residue in another member of the cytokine receptor family.

Alternatively the agent may be capable of binding portions of inhibiting CRD or switching CRD at an interface between the inhibiting CRD and the switching CRD.

In a second aspect the invention could be said to reside in a method for isolating a therapeutic agent said agent being a receptor of the cytokine receptor family having an extracellular receptor module (CRM) composed of two discrete folding domains (RD), a first of the two CRDs being a switching CRD and a second of the CRDs being an inhibiting CRD said CRM molecule being in an inactive state unless activated by ligand, said agonist property being a result of a change in the switching CRD from its inactive state to an active state leading to a signal being produced to effect said agonist property

said method including the steps of contacting candidate agents with fragments of the inhibiting CRD or the switching CRD fragments, assaying candidate agents for their capacity to bind said inhibiting CRD or switching CRD fragments and testing for agonist properties.

The method may including fixing the fragments to a substrate, contacting the fragments with the candidate agents, washing away unbound material not bound to the substrate including unbound candidate agents, separating the bound candidate agents from the fragments or said substrate, identifying the bound candidate agents, testing the agents for antagonist properties.

Such fragments may include any sugar moieties that may be associated with the inhibiting CRD or switching CRD or alternatively may have no sugar moieties attached.

In a third aspect the invention could be said to reside in method of treating a condition in a human or an animal, by administering an agonist of a member of the cytokine receptor family as described or defined herein in a pharmaceutically acceptable form in a suitable carrier, and in a therapeutically effective dose.

It may be desired to treat the condition with one or more of the therapeutic agents identified or defined herein in combination, and perhaps in combination with other therapeutic agents.

The treatment may be aimed at being preventative by reducing the risk of contracting the condition, or the treatment may be used to alleviate or obviate the condition. The administration of the therapeutic agent can be in any pharmaceutically acceptable form in a suitable carrier, and in a therapeutically effective dose.

Agent may be administered in any approved way known the person skilled in the art, or text that might be used such as "Remington's Pharmaceutical Science", Sixteenth Edition, Mack Publishing Company 1980, as a pharmaceutical composition.

Conditions that might be treated include all the conditions currently treated by GM-CSF, IL-3 and IL-5 (e.g. bone marrow depression after chemotherapy, stem cell transplants, *ex vivo* growth of stem cells into mature cells) as well as those that use TPO, G-CSF and other member of the family of Haemopoietic growth factors.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Is a schematic illustration of hβc showing the two cytokine receptor modules (CRMs), the conserved cysteine residues (thin vertical lines) and the characteristic WSXWS motifs (thick vertical lines), also shown are the transmembrane domain (hatched) and the minimal region essential for proliferative signalling (heavier shading), the positions in the hβc cDNA of the XhoI and BglII sites that delimit the fragment used for mutagenesis are shown underneath,

Figure 2A. is a histogram showing proliferation of FDC-P1 cells infected with hβc mutants in the presence and absence of murine GM-CSF, the proliferation assays being carried out in the presence or absence of mGM-CSF for uninfected FDC-P1 cells and cells infected with wild-type hβc ('wt') or the indicated hβc mutants,

Figure 2B. is a histogram showing proliferation of FDC-P1 cells infected with activated hβc mutants that were selected prior to assay by growth in factor-free medium, proliferation of uninfected FDC-P1 cells in the presence of mGM-CSF is shown for comparison,

Figure 3. is a histogram of the proliferation of cells expressing the truncated forms of hβc infected FDC-P1 cells grown in the presence (hatched) or absence (black) of mGM-CSF, aligned beneath is an indication of the extent of the deletions, from left to right being contact with no receptor, with hβcFIΔ, hβc, hβcΔN, hβcΔQP, hβcΔH, hβcΔWS.

Figure 4. is a graphic illustration of a model for hβc activation. It is speculated that activation of hβc via extracellular mutation may involve interaction with an unknown receptor subunit or with a second hβc molecule. Activating mutations or truncation are presumed to unmask interactive residues in domain 4 and lead to signalling in the absence of ligand. Ligand activation, shown on the left may occur through a similar process. Activation through small molecules is also indicated (below).

Figure 5 Molecular modelling of domain 4 of hβc and sequence alignment with other cytokine receptor subunits. Cartoon of Ile^{374} and proposed neighbours. The model of hβc is represented in cartoon form, using Molscript (Kraulis, 1991) and Raster3D (Merrit and Murphy, 1994), viewed from two orientations related by a 180° rotation about a vertical axis. β-strands are indicated by arrowed ribbons and italicized

letters. Residue α-carbon atoms and side-chains are represented by CPK spheres with Ile^{374} being dark and other residues lightly shaded.

Figure 6 Sequence alignment of regions encompassing the proposed B, C and F strands of hβc with equivalent regions of other human cytokine receptor subunits. The predicted extent of each of the indicated β-strands is delimited by the double-headed arrow at top. Conserved hydrophobic residues are boxed and residues in hβc that were targeted for substitution are numbered.

Figure 7A. Analysis of factor independent FDC-P1 cells expressing activate Ile^{374} mutants. Proliferation assay of factor independent FDC-P1 cells selected for growth in the absence of factor. 10^3cells were plated in triplicate and cell proliferation was measured at each time point. The mean and standard error of each triplicate is shown

Figure 7B. Proliferation of factor independent FDC-P1 cells infected with activated hβc mutants. Proliferation assay of FDC-P1 cells, infected with the indicated hβc mutants, which had been selected prior to assay for growth in the absence of factor. Also shown are uninfected cells that were washed and assayed in medium without mouse GM-CSF. The inset shows an enlargement of the proliferation profiles of uninfected cells and cells expressing L356A and W358F mutants. Methods used are as for Figure 7A.

Figure 8 Proliferation of factor independent FDC-P1 cells infected with double mutants derived from I374F. For comparison analyses of uninfected FDC-P1 cells, cells infected with parental I374F and the strongly-activated I374N mutants are also shown. Shown is a proliferation assay of FDC-P1 cells infected with indicated mutants which had been selected prior to assay for growth in the absence of factor. Methods used are as for Figure 7A.

Figure 9 Model for the involvement of interactions between β-strands B and C of domain 4 in receptor activation. (A) In the inactive ie uncomplexed form, interactions (double arrow) between the two β-sheets, comprising β-strands A, B and E (β-sheet 1) and β-strands C, D, F and G (β-sheet 2), respectively, stabilize the inactive conformation of domain 4. (B) Interaction with α subunit plus ligand induces a conformational change in β-sheet 1 that is transmitted via the B-C interaction to generate a conformational change in β-sheet 2; the altered conformations are represented by increased curvature (compared to A). The altered conformation of β-sheet 2 results in association with a second signalling subunit (either another β subunit

- β' - , and as yet unknown signalling subunit, or a putative "γ subunit" - and thus triggers receptor signalling. Note that for the sake of clarity, the ligand itself is not depicted. (C and D) Activating mutations in β-strands B or C (depicted by asterisks *) disrupt interactions between the two β-sheets and result in sheet 2 assuming an activated conformation, which in turn allows interaction with the second signalling subunit (as in part B).

Figure 10 A Structure of hβcFIΔ. A schematic illustration of hβcFIΔ, showing 2 cytokine receptor modules, CRM1 and CRM2 (Goodall *et al*, 1993), which make up the extracellular domain. The transmembrane domain (TM) and intracellular region are also shown. Thick lines indicate WSXWS (Bazan, 1990) motifs. The region duplicated in hβcFIΔ is expanded to show the two repeats. The approximate position of the highly conserved sequence motifs is indicated.

Figure 10 B Structure of hβcFIΔ. Amino acid sequence across the two 37 amino acid repeats (boxed) in hβcFIΔ. Repeat 2 begins after amino acid A431, the original sequence resumes with amino acid R432. Conserved sequences in this region that were targeted for mutagenesis are shaded. The beginning of the TM domain is underlined.

Figure 10 C Structure of hβcFIΔ. Summary of deletions introduced into the repeats of hβcFIΔ. Δ1 was introduced into both repeats separately (Δ1-R1 and Δ1-R2) while Δ2 was only introduced into repeat 2 (Δ2-R2).

Figure 10 D Structure of hβcFIΔ. Sequence of the hβcHSV insertion mutation. The inserted epitope is boxed.

Figure 11A Analysis of hβc containing an 11 residue insertion after position 431. Shown is a histogram representing the results of proliferation of G418 resistant FDC-P1 cell populations infected with pRufNeo or pRUFNeo encoding hβcFIΔ or hβcHSV in the presence (black boxes) or absence (white boxes) of mGM-CSF. The Y-axis shows absorbance at 570 - 655nm.

Figure 11B Analysis of hβc containing an 11 residue insertion after position 431. Shown is a histogram representing the results of proliferation of factor-independent FDC-P1 cell populations. After infection with pRufhβcFIΔ or pRufhβcHSV, cells were selected in the absence of growth factor. Proliferation was assayed in the presence (black boxes) or absence (white boxes) of mGM-CSF.

Figure 12. Proposed model of active and inactive structures of hβc. Only the predicted structure for the membrane proximal domain of hβc and hβcFIΔ is shown. The structure of hβc is predicted on the basis of the known growth hormone receptor structure (deVos *et al* , 1992). A) *hβc domain 4.* β-strands are labelled A-G. The position of the WSEWS sequence is indicated (WS). B) *hβcFIΔ domain 4.* In hβcFIΔ repeat 1 is predicted to fold into a normal domain 4 structure with repeat 2 looped out at the insertion point between the F' and G' β-strands. Repeated β-strands (E1, F1, E2, F2) and WSEWS sequences (WS1 and WS2) are indicated. In hβcFIΔ we suggest that the extruded sequences from repeat 2 interfere with an inhibitory interaction that normally maintains the receptor in an inactive state. In the presence of mutations in repeat 2, the repeat 1 sequence maintains its position incorporated in the domain 4 structure. C) *Repeat 1 mutations.* In the presence of mutations to the RVRVR and WSEWS sequences of repeat 1 we speculate that sequences from repeat 2 can substitute in an alternative structure for domain 4. It seems most likely that sequences from repeat 1 will extrude from the normal structure after the E1 β-strand. In this alternative structure we predict that the excluded sequences no longer induce activity and the receptor functions identically to the wild type form of hβc in response to IL-3. D) *Possible structure for the 11 residue insertion.* The 11 residue sequence is shown extruded at the insertion point in a conformation similar to hβcFIΔ. The excluded sequence is predicted to interfere with an inhibitory interaction inducing activation.

DETAILED DESCRIPTION OF THE EXAMPLES.

EXAMPLE 1

CONSTRUCTION AND TESTING OF ACTIVATION POINT MUTANTS IN THE COMMON β SUBUNIT

Cell lines and cDNAs

PA317 (Miller and Buttimore, 1986) and Ψ2 (Mann *et al.*, 1983) retrovirus packaging cell lines were maintained in DME medium supplemented with 10% foetal calf serum. The IL-3/GM-CSF-dependent mouse myeloid cell line, FDC-P1 (Dexter *et al.*, 1980), was maintained in the same medium, as above, containing 80 units/ml mouse GM-CSF (produced by an engineered yeast strain and kindly supplied by Dr Tracy Wilson, Walter and Eliza Hall Institute, Melbourne) or 300 units/ml mouse IL-3 (produced from a baculovirus vector and kindly supplied by Dr Andrew Hapel, John Curtin School of Medical Research, Canberra), respectively.

The hβc cDNA (Hayashida *et al.*, 1990) used here was that described by Barry *et al.* (1994). cDNA for hGMRα (Gearing *et al.*, 1989) was kindly provided by Dr N. Nicola (Walter and Eliza Hall Institute, Melbourne, Australia). The activated FIΔ mutant of hβc has been described previously (D'Andrea *et al.*, 1994).

Generation of a retroviral expression library of point-mutated hβc cDNA constructs

Point mutations were randomly introduced into a 940bp segment of the hβc cDNA encoding the 191 membrane-proximal residues of the extracellular domain, the transmembrane domain and the first 92 residues of the cytoplasmic domain. We identified this segment as a potential target for activating mutations because it encompasses several sequences that have been implicated in signalling and/or activation of cytokine receptors: (i) the membrane-proximal cytoplasmic region essential for signal transduction (Sakamaki *et al.*, 1992; Sato *et al.*, 1993; Ziegler *et al.*, 1993, D'Andrea *et al.*, 1991), (ii) the highly conserved WSXWS sequence that is a hallmark of the cytokine receptor superfamily (Bazan, 1990), (iii) the equivalent extracellular region of c-Mpl remaining in the v-Mpl oncoprotein (Souyri *et al.*, 1990; Vigon *et al.*, 1992), and (iv) the extracellular sequence duplicated in the constitutively active hβc mutant described by D'Andrea *et al.* (1994).

The bank of random point mutants was insert *en masse* into a retroviral expression vector. The resultant retroviruses were then used to infect a murine factor-dependent haemopoietic cell line (FDC-P1), following which these cells were selected for the ability to grow in the absence of factor. This procedure is essentially a combination of the PCR mutagenesis technique described by Cadwell and Joyce (1992) and methodology previously developed in this laboratory (Rayner and Gonda, 1994).

We were able to define PCR conditions, based on those described by Cadwell and Joyce (1992), that resulted in the unbiased, random generation of mutations at the desired rate of approximately 0.2% (1 in 500bp). The mutated hβc fragments were directionally inserted into pRUFNeo-hβc from which the segment subjected to PCR mutagenesis had been excised. This resulted in the generation of a library, comprising 1.7×10^5 plasmid clones, of hβc cDNAs bearing point mutations in the targeted segment only ("$h\beta c^{mut}$"). Using procedures described previously (Rayner and Gonda, 1994) the plasmid DNA was used to generate a retroviral library estimated to contain 2.1×10^4 independent viral producer clones. Assuming that there was no overwhelming bias in the procedure, this should adequately represent all of the possible point mutations in the 940bp hβc fragment.

Isolation of factor-independent FDC-P1 cells carrying constitutively active hβc mutants

FDC-P1 cells are dependent on mouse GM-CSF (mGM-CSF) or IL-3 (mIL-3) for growth, and normally die within days when starved of either factor. However, they do proliferate in response to low concentrations of human GM-CSF (hGM-CSF) or IL-3 (hIL-3) if the appropriate a subunit (hGMRα or hIL-3Rα) is co-expressed with hβc (our unpublished results). We therefore reasoned that infection of FDC-P1 cells with a retrovirus encoding a constitutively active form of hβc should induce factor-independent proliferation; this approach has been validated by the recent isolation of a constitutively active hβc mutant, FIΔ, expressed in factor-independent FDC-P1 cells (D'Andrea *et al.*, 1994).

Thus, to isolate factor-independent cells expressing constitutively active hβc point mutants present in the hβc expression library, FDC-P1 cells were infected by co-cultivation with the pool of $h\beta c^{mut}$ virus-producing Ψ2 cells. Parallel co-cultivations were also performed with uninfected Ψ2 cells and Ψ2 cells producing wild-type hβc retrovirus. After one week in liquid culture in the absence of factor, all four FDC-P1 cell pools infected with the mutant hβc retroviral library contained viable, proliferating cells while no such cells were present in the control cultures. Factor independence was not a result of autocrine growth factor production as conditioned medium from the factor-independent cell pools did not support the growth of uninfected FDC-P1 cells (data not shown). A total of 31 factor-independent clones was isolated from the factor-independent liquid culture pools by agar plating; each of these was analysed further. Based on infection frequency and the number of independent mutants (detected by sequencing and/or restriction analysis), we estimate the frequency of factor-independent mutations to be about 1 in 10^5.

Identification of activating mutations in constitutively active hβc mutants

During the course of recovering the mutated region of hβc from the factor-independent clones by PCR, it was discovered that 22/31 clones contained an additional BglII restriction site. Sequencing of one such clone (mutant 1) revealed that this was due to a T to A mutation at nucleotide 1374 which results in a change of the amino acid valine to glutamic acid at residue 449 (V449E) within the transmembrane domain. This is strikingly reminiscent of the activating point mutation (V664E) found by Bargmann *et al.* (1986) in the rat *neu* oncogene (see Discussion). Sequence analysis of one other clone that lacked the extra BglII site (mutant 2) revealed two point mutations: an A to G substitution at nucleotide 1112 which resulted in the substitution of lysine 362 to glutamic acid (K362E), and a T to A substitution at nucleotide 1149 which resulted in the substitution of isoleucine 374 to asparagine (I374N), both of which lie in the

extracellular portion of the targeted region. The latter mutation results in the loss of a BstYI restriction site; by digesting with BstYI, we subsequently found that all 9 of the factor-independent clones that lacked the V449E mutation carried the I374N mutation (data not shown).

To confirm that the V449E mutation was indeed capable of activating hβc, and to identify which of the two mutations in mutant 2 was responsible for activation, all three mutations were independently re-created by site-directed mutagenesis. Following insertion into the pRUFNeo retroviral vector and transfection into Ψ2 packaging cells, these mutants, as well as wild-type hβc, were again introduced into FDC-P1 cells, which were then selected either for G418 resistance or for growth in medium without mGM-CSF. All of the viruses efficiently generated G418-resistant cells, which were subsequently analysed for hβc expression by antibody staining and flow cytometry. In these and subsequent experiments we also included the previously-described activated hβc mutant FIΔ, which contains a 37 amino acid duplication in the membrane-proximal portion of the extracellular domain (D'Andrea *et al.*, 1994). However, only the I374N, V449E and as expected, FIΔ mutants conferred factor independence on the FDC-P1 cells. Neither the K362E mutation nor an additional mutation V449D were able to confer factor independence.

Although the degree of proliferation in the absence of factor shown by cells infected with the V449E, I374N and FIΔ mutants was lower than that seen with factor, this probably reflects the fact that only a proportion of each G418-selected population expressed hβc. When these same G418-resistant populations were subsequently selected for factor independence, the proliferation observed in the absence of factor was comparable to that of uninfected FDC-P1 cells in the presence of mGM-CSF (Figure 2B). We therefore concluded that the V449E and I374N substitutions could confer factor independence on FDC-P1 cells and thus could constitutively activate hβc.

The possible mechanisms by which the extracellular mutants might act are discussed in examples 2, 3, and 4

EXAMPLE 2

EXTRACELLULAR TRUNCATION TO DEFINE A MINIMAL FUNCTIONAL EXTRACELLULAR DOMAIN OF THE COMMON SIGNALLING SUBUNIT FOR GM-CSF, IL-3 AND IL-5.

Generation of hβc mutants.

We used *in vitro* mutagenesis to construct the series of extracellular deletion mutants shown in Figure 3. In each of these mutations a large extracellular sequence has been deleted extending from the residue after the introduced N-terminal FLAG™ octapeptide to various residues within the membrane proximal cytokine receptor module (CRM). The N-terminal FLAG octapeptide does not affect receptor function (data not shown) and potentially allows detection of surface expression of altered receptors. The mutant hβcΔN completely removes the N-terminal CRM (residues E25-E232 inclusive) while the mutant hβcΔQP removes the N-terminal CRM and the first domain of the second CRM (residues E25-N337 inclusive). hβcΔH is truncated to a site within the membrane proximal domain (residues E25-A394 deleted) leaving behind sequences which are duplicated in the constitutive hβc mutation reported previously (D'Andrea *et al.*, 1994) and retained in the truncated, constitutively active form of TPO receptor, *v-mpl.* The mutant hβcΔWS removes both CRM's (including the membrane proximal WSXWS ; deletion of residues E25-A431) and leaves only 7 residues between the FLAG octapeptide and the transmembrane domain. These modified hβc cDNAs were cloned into the retroviral expression vectors pRUFNeo (Rayner *et al.*, 1994) or pRUFPuro (Jenkins *et al.*, 1995) and introduced into the retroviral packaging cell line, ψ2. Stable pools of transfected cells were produced by antibiotic selection. To test the ability of each mutant to induce factor independent growth we infected the murine myeloid cell line FDC-P1 and the IL-3 dependent pro-B cell line BaF-B03. Following antibiotic selection cells were assayed for the ability to proliferate in the presence and absence of the relevant growth factor or for their capacity to bind hIL-3. To test ligand responsiveness mutant receptors were introduced into an IL-2 dependent mouse T-cell line expressing the hIL3Rα subunit from a retroviral insert (CTL-EN/hIL3Rα).

Proliferation of cells expressing hβc mutant receptors.

G418-selected populations of FDC-P1 cells were assayed for their ability to proliferate in the presence and absence of murine GM-CSF. Cells expressing two mutant forms of hβc were included as controls : hβcFIΔ, expression of which generates factor independence in FDC-P1 cells but not Ba/F3 cells (D'Andrea *et al* 1994; Jenkins *et al*, 1995) and hβcV449E, expression of which generates factor independent FDC-P1 and Ba/F3 cells (Jenkins *et al*, 1995). Uninfected FDC-P1 cells and FDC-P1 cells infected with the hβcΔN and hβcΔWS retrovirus grew in the presence of mGM but failed to grow after removal of growth factor. FDC-P1 cells infected with either the hβcΔH or hβcΔQP deletion mutants grew in the absence of growth factor (see figure 3).

Discussion

We expressed truncated forms of hβc in the murine IL-3/GM-CSF dependent cell line, FDC-P1 and measured their proliferation potential. Expression of two of the truncated receptor mutants, hβcΔQP and hβcΔH, led to factor independent growth in these cells. We confirmed cell surface expression of the truncated receptors via the introduced FLAG epitope in the FDC-P1 cells. The observation that hβcΔH confers factor independence while hβcΔWS does not suggests that a region of hβc (residues H395-A431) is essential for constitutive activation. This membrane proximal segment contains the WSXWS motif and the conserved β strand F'(consensus Y/H XXRVRXR). These sequences are also within a duplicated segment in the mutant hβcFIΔ which led us to speculate that they are involved in receptor activation (D'Andrea *et al.*, 1994).

The activated forms of hβc with extracellular activating mutations (hβcFIΔ, hβcI374N, hβcΔQP and hβcΔH) are consistent with activation involving disruption of an inhibitory conformation in the monomeric, non-ligand bound form of hβc. In this conformation we would predict that critical conserved residues within domain 4 (in the segment H395-A431) are prevented from intermolecular association. The fact that the mutant hβcΔN does not confer factor independence in FDC-P1 cells implies that the third fibronectin like receptor domain contributes to the masking that is preventing signalling. Removal of this domain is sufficient to lead to activation as hβcΔQP is constitutively active. Furthermore, the mutant hβcΔH which only retains part of the membrane proximal domain appears to present the conserved sequences for interaction. This may suggest that this conserved segment can maintain some structure in the absence of other sequences from this domain. We would propose that the I374N point mutation can interfere with the structure of domain 4 in such a way that it leads to the disruption of inhibitory interactions.

EXAMPLE 3

INTERACTING RESIDUES IN THE EXTRACELLULAR REGION OF THE COMMON β SUBUNIT OF THE HUMAN GM-CSF, IL-3 AND IL-5 RECEPTORS INVOLVED IN CONSTITUTIVE ACTIVATION.

We have used sequence alignment of the extracellular region of hβc with hGHR and other members of the cytokine receptor family, and comparison with the structure of the hGH-hGHR complex, to derive a molecular model of the membrane-proximal CRM of hβc. A model of the fourth domain of hβc was developed based on the crystal structure

coordinates of the human growth hormone binding protein (GHbp) (De Vos *et al* , 1992). The sequences of hβc and domain 2 of the GHbpII were aligned manually and an Indigo computer (Silicon Graphics) was used to run the molecular modelling programs Insight II, Homology and Discover (Molecular Simulations Inc., San Diego, CA). Coordinates for regions of hβc thought to be conserved structurally, corresponding to the proposed β-strands, were assigned from the homologous backbone coordinates of GHbpII and some side-chain coordinates. Additional loops were assigned from coordinates from a library of protein structures. An inspection of the model revealed a well-packed hydrophobic core with only moderate steric clashes between the hydrophobic side-chains of adjacent strands. Manual and automated methods were used to select appropriate conformations for the hydrophobic side-chains of residues proposed to be buried in the core of the hβc molecule. The model was evaluated for stereochemical parameters using Procheck (Laskowski *et al*, 1993). Figure 5 shows the predicted structure of the membrane-proximal subdomain (β4) of hβc.

Biological activity of Ile^{374} hβc mutants expressed in FDC-P1 cells. As shown in example 1 the substitution of asparagine for isoleucine at residue 374 (I374N) in domain 4 of hβc resulted in constitutive activation as shown by its ability to confer factor-independent growth on the factor-dependent haemopoietic cell line, FDC-P1. The model illustrated in Figure 5 suggests that the Ile^{374} residue lies on the C strand and is buried within a hydrophobic region in domain 4 of hβc. Furthermore, sequence alignment of the extracellular region of hβc with hGHR and other members of the cytokine receptor superfamily suggest that the hydrophobic nature of the Ile^{374} residue in hβc is highly conserved at the corresponding positions amongst members of this receptor family (Figure. 6). Based upon these observations, it is likely that the hydrophilic substitution in I374N would severely distort the conformation of this region.

To further examine the structural requirements for receptor activation at position 374, site-directed mutagenesis was used to introduce several different amino acids at this position. Aspartic acid (I374D) and glutamine (I374Q) substitutions were chosen, based on size and structure, as those most likely to mimic the conformational disruption induced by the hydrophilic substitution in I374N, whereas the large phenylalanine residue (I374F) was used to investigate the effect of a possible steric disruption, while maintaining hydrophobicity at the position. Substitution by the small alanine residue (I374A) was used to test whether receptor activation would result from a mutation which is predicted to be minimally disruptive. The Ile^{374} hβc mutant cDNAs were

inserted into the pRUFNeo retroviral vector and introduced into FDC-P1 cells, which were then selected for G418 resistance. Flow cytometric analysis of G418-selected FDC-P1 cells using standard indirect immunofluorescence indicated that a significant proportion of infected cells expressed each Ile^{374} hβc mutant on the cell surface.

The ability of each mutant to constitutively generate a proliferative signal was determined by analyzing the infected cells for factor-independent growth. All of the substitutions at position 374, except alanine, were able to confer factor independence on the FDC-P1 cells (not shown).

To compare the efficiencies with which each mutant could activate hβc, G418-resistant cells were selected for factor independence in liquid culture. Interestingly, in several independent experiments, the proliferation rate of factor-independent cell populations infected with the I374F mutant was consistently slower than that of cells infected with the I374D, I374Q and I374N mutants (Figure 7A), although surface expression was similar in each case (not shown).

Constitutive activation of wild-type hβc upon substitutions at Leu^{356} or Trp^{358} residues. We used a molecular model of domain 4 of hβc (Fig. 1A) to identify the residues within this domain of hβc most likely to interact with Ile^{374}. Using this approach, four residues - Leu^{356}, Trp^{358}, Val^{412} and Val^{414} - were predicted to interact with Ile^{374}. The two Val residues are located on the F strand in the same β-sheet as Ile^{374} (on strand C), whereas the Trp and Leu residues are located on the B strand in the opposing, second β-sheet (Figure 5). Interestingly, sequence alignment of the extracellular regions of hβc and other members of the cytokine receptor family indicate that the Trp^{358} residue is highly conserved amongst other cytokine receptors. These alignments also indicate that the hydrophobicity of residues corresponding to the Leu^{356} position is conserved in other cytokine receptors, and that the Val^{412} and Val^{414} residues are part of a conserved motif - "RVRVR" in hβc - typified by an Arg-Val-Arg sequence (D'Andrea *et al*, 1990; Pathy, 1990) (Figure 6).

We reasoned that if, as predicted, the residues at positions 356, 358, 412 and 414 normally interacted with Ile^{374}, then substitutions at some or all of these positions might also disrupt these interactions and result in receptor activation. Since strong activation occurred by replacing the hydrophobic Ile^{374} residue with asparagine, we first tested the effect of this substitution at each of the potentially interacting positions to generate the mutants L356N, W358N, V412N and V414N. Retroviruses encoding these mutants, as well as wild-type hβc, were used to infect FDC-P1 cells which were then selected for G418 resistance in liquid culture. Initial flow cytometric analyses of the resultant

cell populations indicated that cell-surface expression of some hβc mutants was only detectable by high-sensitivity immunofluorescence (data not shown); as a result, subsequent flow cytometric analyses were performed on cells stained by this method (see Experimental Procedures). After several weeks' selection for factor independence in liquid culture, only cultures infected with the Leu^{356}, Trp^{358} and, as expected, the Ile^{374} mutants contained viable, proliferating cells (Figure 6B and data not shown). To test the effects of potentially less severe disruptions (akin to I374F), we introduced other, hydrophobic residues at positions 356 and 358 by substituting alanine for Leu^{356} (L356A) and phenylalanine for Trp^{358} (W358F). Following introduction into FDC-P1 cells, it was seen that these mutants were expressed on the cell surface (not shown); subsequent culture in the absence of mGM-CSF showed that both induced weak but detectable (Figure 7B) factor-independent growth.

The rate of factor-independent proliferation of cells expressing the L356N and W358N mutants was considerably lower than that seen with I374N, as shown by proliferation assays (Figure 7B). Furthermore, the proliferation rates of factor-independent cell populations infected with the L356A and W358F mutants were several-fold lower even than those of cells infected with the L356N and W358N mutants, although surface expression was slightly higher for the former two mutants (Fig. 5B). Interestingly, the factor-independent colonies that arose from FDC-P1 cells infected with the L356A and W358F mutants were significantly smaller than those infected with the other activated mutants (data not shown). Together, these results suggest that the Leu^{356} and Trp^{358} mutants induced constitutive activation less efficiently than the Ile^{374} mutants and that the asparagine substitutions at these positions lead to higher activity than the more hydrophobic (alanine and phenylalanine) substitutions.

Substitutions at positions 356 or 358 can synergistically enhance activation of the I374F mutant. An extension of the notion that disruption of interactions between Ile^{374} and Leu^{356}/Trp^{358} leads to constitutive activation is that weakly activating mutations at both of the interacting positions might synergise in enhancing receptor activation. We therefore constructed four double mutants by combining I374F with L356A, W358F, and also with glycine substitutions for each of the two valine residues at positions 412 and 414. The two latter double mutants (I374F/V412G and I374F/V414G) were constructed to provide negative controls for synergy as asparagine substitutions alone for Val^{412} or Val^{414} did not result in activation. All four double mutants conferred factor independence upon FDC-P1 cells; however, the proliferation rates of factor-independent cells infected with the I374F/L356A and I374F/W358F mutants were consistently several-fold higher than those infected with the I374F/V412G, I374F/V414G or I374F mutants (Figure 8).

Indeed, the proliferation rates seen with the I374F/L356A and I374F/W358F mutants were similar to that of the strongly-activated I374N mutant. In contrast, the proliferation rates of cells expressing the I374F/V412G and I374F/V414G mutants barely differed from that of cells expressing the "parental" I374F single mutant. Differences in growth rates could not be attributed to corresponding differences in the level of cell-surface expression of the various mutants. Thus these data, and the data of Figure 7A and B, are consistent with the notion that activation of hβc by the Ile^{374}, Leu^{356} and Trp^{358} mutants is due to disruption of the interactions between Ile^{374} and the latter two residues.

Discussion

As one way of exploring the mechanism by which the I374N mutation (and possibly other mutations in domain 4), acts, we have utilised a molecular model of part of the extracellular portion of hβc to design further mutants. In particular, we have focussed on possible interactions between Ile^{374} and other, neighbouring residues in the predicted hβc structure.

One of the key observations in this work was that replacement by asparagine of Trp^{358} or Leu^{356}, which are predicted to participate in Van der Waals interactions with Ile^{374}, also resulted in activation. Potentially less disruptive mutants, in which these residues were replaced with phenylalanine or alanine (in W358F and L356A, respectively), were very weakly activating by themselves. However, we found that these relatively mild changes greatly enhanced factor-independent proliferation when combined with a relatively weak mutation (I374F) at position 374. The results support the prediction that Ile^{374} interacts with Leu^{356} and Trp^{358}, and lead us to suggest that (i) these interactions are normally involved in maintaining the conformation of domain 4, and (ii) that disruption of these interactions leads to a conformational change which results in receptor activation. There are several other observations that support this interpretation of our results. First, substitutions at position 374 other than the original asparagine resulted in activation, with those expected to be most disruptive, ie other hydrophilic residues, resulting in maximal activation as judged by the growth rates of factor-independent cells. Secondly, a similar pattern holds for substitutions at positions 356 and 358 in that the asparagine substitutions induced far greater factor-independent growth than the alanine (L356A) or phenylalanine (W358F) substitutions. Third, we note that the interacting residues Ile^{374} and Trp^{358}/Leu^{356} are predicted to lie on β-strands C and B, respectively, and so we could generalise, with support from the molecular model, that other interactions between these two strands may also be important in maintaining the normal structure of domain 4. Indeed, preliminary data from random mutagenesis studies suggest that Tyr^{376} in strand C is a target for

activating mutations. Finally, the fact that like I374N, neither the W358N nor the L356N mutant could confer factor independence on BAF-B03 cells is consistent with a common mode of action.

Structural modelling of the α-β-ligand complex indicates that domain 4 of hβc can be viewed as two β-sheets, one comprised of strands A, B and E (β-sheet 1), and the second comprised of strands D, C, F and G (β-sheet 2) (DeVos *et al* , 1992; Goodall *et al*, 1993). The model, which is supported by extensive studies on the interactions between GM-CSF (and IL-3) and both the α and β subunits (eg (Bagely *et al*, 1995)), predicts that β-strand E and the A-B loop in β-sheet 1 contact the α subunit (see Figures. 5 and 9). Thus, we would predict that interactions with a second signalling subunit (either a β or a 'γ' subunit) would take place *via* the opposite "side" of domain 4 ie β-sheet 2 (see Figure 9), which contains, as has been previously mentioned (Bazan, 1990; D'Andrea *et al*, 1994; Pathy, 1990) the conserved "WSXWS" and "RVRVR" motifs. In view of this and the results presented in this report, we propose the following model, illustrated in Figure 9, for the role of domain 4 in the activation of hβc. In both inactive and active forms of the wild-type receptor, β-sheets 1 and 2, and specifically strands B and C interact *via* contacts including those between Ile^{374} and Trp^{358}/Leu^{356} . Association with the α subunit plus ligand induces a conformational change in β-sheet 1 or a rearrangement of the interface between the two sheets, and this is transmitted *via* the B-C interaction to the second β-sheet. The ensuing conformational change (to which contacts between ligand and hβc could also contribute) then promotes interaction of residues in this β-sheet with the second signalling subunit, leading to dimerization and triggering of intracellular signalling pathways. In the case of the activating mutations affecting Ile^{374}, Trp^{358} and Leu^{356}, disruption of the B-C interaction would lead to the second β-sheet assuming an activated conformation similar to that seen after α subunit/ligand binding in the normal receptor.

We speculate that the residues involved in interactions with a second signalling subunit may include a conserved structure comprising the WSXWS and RVRVR motifs. This is consistent with the observation that a duplication within hβc which includes these motifs induces constitutive activation (D'Andrea et al, 1994), possibly by unmasking one copy of the interacting structure. It is also consistent with our preliminary observations that while other (potentially) activating *point* mutations in domain 4 are clustered around β-strands B and C, none have been found to date in or between strands F and/or G. Validation or rejection of this model will ultimately require definition of the subunit composition of both wild-type and mutant GMR/IL-3R/IL-5R complexes, and identification of all surfaces participating in inter-subunit interactions.

Finally, we note that the three interacting residues studied in this report - Ile^{374}, Leu^{356} and Trp^{358} - are highly conserved within the cytokine receptor family (eg see Figure 6). Thus, the homologous residues in other cytokine receptors may be targets for activating mutations and, furthermore, the model proposed here may also be applicable to other receptors.

EXAMPLE 4

MUTAGENESIS OF AN ACTIVATING DUPLICATION IN DOMAIN 4 OF Hβc.

Structure and origin of hβcFIΔ

The activated hβc mutant, hβcFIΔ, arose spontaneously after retroviral infection of the murine myeloid cell line FDC-P1 with a hβc retroviral construct (D'Andrea *et al* , 1994). While this mutant confers ligand-independence upon FDC-P1 cells and primary myeloid progenitors it behaves identically to wild type hbc in CTLL or BaF-B03 cells (Jenkins *et al* , 1995) (M. McCormack and T. J. Gonda, in preparation). The structure of hβcFIΔ is shown in Figure 10. It contains a 111bp duplication that leads to a 37 amino acid duplicated segment in the membrane proximal domain (domain 4) of hβc, including the WSEWS sequence and a conserved, alternating aliphatic/basic segment that corresponds to the predicted β-strand F'. The duplicated region corresponds exactly to a minimal segment required for constitutive hβc activation by extracellular truncation (D'Andrea *et al* , 1996).

Design and introduction of mutations

We wished to define the duplicated sequences required for signalling by hβcFIΔ and, more specifically, to test whether duplication of the conserved motifs was important for activation. In the absence of structural data for hbc and given the absence of mutagenic studies across this region we were also interested to examine the role of these conserved motifs in the context of the IL-3-dependent function of hβc and hβcFIΔ.

The hβcFIΔ cDNA was cloned into pALTER and non-conservative substitutions were introduced, separately, into each copy of the duplicated segments (summarised in table 1). The WSXWS motif has been reported to be intolerant to mutation and appears to have a role in forming a scaffold that is important for correct folding and transport of the receptor (Hilton *et al* , 1995). A mutation (WSEWS->SSESS) was introduced that when tested in another receptor, IL2Rβ, permitted expression on the cell surface (Miyazki *et al* , 1991). Since hβcFIΔ contains two copies of WSEWS in the second cytokine receptor module (CRM2), the mutants will still retain one intact copy of the

WSEWS sequence in this domain and thus may be better able to maintain structural integrity and be transported to the cell surface. We also targeted the tyrosine residue at the beginning of predicted β-strand F', replacing it with glycine. This position exhibits strict conservation across the receptor superfamily, being nearly always occupied by a tyrosine or histidine residue. The conserved sequence corresponding to β-strand F' (consensus Y/H XX R/Q VRXR) has not been analysed in any detail in hβc or other receptors of this family. A double mutation which was designed to cause maximal disruption to any associated function was introduced into this strand. This placed a glutamic acid residue into a normally buried position and an alanine in place of a conserved arginine residue (RVRVR->RERVA). In order to analyse the role of these conserved motifs in the context of a normal receptor response to ligand we have introduced identical amino acid substitutions into the wild type hβc cDNA (Table 2).

In addition to these substitutions, deletions were introduced into hβcFIΔ that remove approximately half of the duplicated segment (Figure. 10C). Deletion 1 (Δ1) removed the C-terminal half of the duplicated segment (either repeat 1 or 2) including the WSEWS sequence. On the basis of structural predictions and sequence alignments with other receptors ((Goodall *et al*, 1993) and C. Bagley, unpublished) this deletion was designed to remove a region of the duplication including the F' and G' β-strands and the intervening loop. Two serine residues were introduced to partially bridge the remaining segments of β-strands F' and G'. Deletion 2 (Δ2; repeat 2 only) removes the N-terminal half of the duplicated segment. Altered hβcFIΔ and hβc cDNAs were cloned into retroviral vectors and the resulting retrovirus introduced into FDC-P1 cells or CTL-EN/hIL3Rα cells for analysis. Results are summarised in tables 1 and 2.

<u>Effects of mutations on the expression and function of hβc and hβcFIΔ in FDC-P1 cells.</u>

The two double amino acid substitutions that were introduced into the conserved motifs of the wild type hβc (RV-wt and WS-wt) abolished detectable surface expression on FDC-P1 and ψ2 producer cells (assayed using a sensitive three layer detection protocol). This is consistent with a role for this segment in receptor transport, folding or processing. Not surprisingly these amino acid substitutions in wild type hβc did not lead to factor independent growth of FDC-P1 cells (data not shown). Whatever the requirement for these sequences in hbc may be, it is abrogated in hβcFIΔ as the corresponding mutations in either repeat (RV-R1, RV-R2, WS-R1, WS-R2) had no adverse affect on expression (Table 3). This is presumably due to the duplication of this segment. In fact a mutant in hβcFIΔ in which both WSEWS sequences were altered (repeat 1 WSEWS->SSEWS and repeat 2 WSEWS->SSESS) was not

expressed, consistent with at least one intact membrane proximal copy of the WSEWS sequence being required in CRM2 for efficient transport and expression (data not shown).

All of the mutations introduced into repeat 1 (apart from the Y-R1 substitution - see below) abolished factor-independent growth in FDC-P1 cells. In contrast, the identical mutations in repeat 2 did not abolish factor-independent proliferation of FDC-P1 cells. To test whether specific sequences, essential for factor-independence, were contained in the N-terminal half of repeat 2 and were therefore not affected by any of our mutations we also generated a deletion removing the first 18 residues of this repeat (Δ2-R2). This mutant was detectable on the surface of FDC-P1 cells and was capable of conferring factor-independent growth in FDC-P1 cells . Thus, none of the substitutions or deletions introduced into repeat 2 affected the capacity of hβcFIΔ to confer factor-independence in FDC-P1 cells. These results suggest that there is little constraint on the sequence or length of the duplication that is able to induce activation.

Substitution of glycine for the conserved tyrosine at the beginning of predicted β-strand F' in either duplicated segment of hβcFIΔ (Y-R1 and Y-R2) had no effect on its ability to induce factor-independence in FDC-P1 cells. In the growth hormone receptor this tyrosine forms a hydrogen bond to the backbone of the E'-F' loop via its hydroxyl group. It is possible that this conservation reflects a subtle structural requirement and that other alterations would be required in combination to impair receptor function.

<u>An unrelated insertion at position 431 of hβc induces constitutive activity in FDC-P1 cells</u>

Two alternative mechanisms may explain the activity of hβcFIΔ; it is possible that duplication of a dimerisation or interaction epitope drives constitutive association with a required cofactor. Alternatively, disruption of an inhibitory interaction may occur due to interference of additional sequences with the native hbc structure. Mutagenesis of repeat 2 suggests little sequence constraint in this repeat and is most consistent with the second alternative. To distinguish between these alternatives we tested the possibility that an unrelated sequence inserted in the same position as repeat 2 of hbcFID could cause activation. We designed an oligonucleotide to introduce the sequence QPELAPEDPED (an 11 amino acid epitope from herpes simplex virus, HSV) into hβc after residue 431. The mutant receptor generated had the sequence QSELAPEDPED inserted at this position (Figure. 10D) suggesting that an error was incorporated during the mutagenesis reaction. However, retrovirus encoding this mutant (hbcHSV) was introduced into FDC-P1 cells and infected cells were selected for G418 resistance. Expression of receptor was confirmed by flow cytometry and was found to be similar to

that of hβcFIΔ. When infected cells were washed growth was detected in the absence of growth factor. Although the level of proliferation observed was significant, it was considerably less than that observed for hβcFIΔ. This suggested that only a subpopulation of the cells expressing the altered form of hβc were capable of growth factor-independent proliferation and/or that this mutation is only partially activating. We then allowed the resultant growth factor-independent cells to expand and examined the hβc expression by flow cytometry . Again, the hβcHSV infected cells displayed similar levels of expression to hβcFIΔ. The reduced level of proliferation is not due to a reduced signalling capacity since in the absence of cytokine the cells selected for growth factor-independence grew at a comparable rate to cells expressing hβcFIΔ. Thus, the HSV insertion is weakly activating and this weak activity is not augmented by having increased levels of expression.

Function of mutant receptors in CTL-EN cells expressing hIL3Rα

The ability of hβcFIΔ to function with ligand in CTLL cells expressing the hIL3Rα subunit (Jenkins *et al*, 1995) permits an independent assay of the effects of introduced mutations on ligand-dependent function. This is important as previous work has suggested that constitutive activity in FDC-P1 cells and the response to ligand in CTLL cells may represent alternative mechanisms of activation (Jenkins *et al*, 1995; D'Andrea *et al* , 1994). This assay is also a useful measure of receptor integrity for mutations that have abolished constitutive activity. We next tested the effect of the mutations that were introduced into wild type hβc, and also assayed the effects of the mutations in hβcFIΔ with respect to their affect on ligand-dependent signalling in these cells.

All altered forms of hβc and hβcFIΔ were introduced into CTL-EN cells expressing the hIL3 specific alpha chain (CTL-EN/hIL3Rα) and assayed for the ability to confer growth in the presence of rhIL-3. Retrovirus carrying mutant receptor cDNA was introduced into CTL-EN/hIL3Rα cells by co-cultivation, cell populations were split in two and either selected for growth in a high dose of hIL-3 (100ng/ml) or were grown in mIL-2 and G418. To assess the efficiency of retroviral infection and to measure receptor expression levels cells grown in mIL-2 and G418 were stained with hβc-specific monoclonal antibodies 2-4 days after infection. Uninfected CTL-EN/hIL3Rα cells did not grow in the presence of hIL-3 at concentrations up to 1000ng/ml. However, infection of these cells with hβc or hβcFIΔ retrovirus generated cells that responded in a dose-dependent fashion to hIL-3 (data not shown).

Retrovirus carrying the RV-wt or WS-wt mutants did not generate viable cells when selected in 100ng/ml hIL3. We could not demonstrate hbc expression using the more

sensitive 3-layer protocol following infection suggesting that these mutations abolished surface expression of hβc (data not shown) as also seen on infected FDC-P1 cells and transfected ψ2 producer cells. After permeabilising cells we can readily detect hbc expression by flow cytometry (data not shown) suggesting an intracellular accumulation of receptor in cells infected with mutant hbc retrovirus. These results are consistent with a role for these sequences in receptor folding and secretion. It has been demonstrated recently that substitutions of serine for tryptophan in the WSXWS motif of mEpoR prevent surface expression and lead to accumulation of this receptor in the endoplasmic reticulum (Hilton *et al* , 1995).

A summary of the hβcFIΔ mutations and their activity in CTL-EN/IL3Rα cells (and FDC-P1 cells) is shown in Table 1. Only the receptor with a deletion of 18 amino acids in repeat 1 (Δ1-R1) failed to mediate hIL-3 dependent growth in these cells even though there was a small but significant shift in fluorescence when receptor expression was analysed by flow cytometry. This mutation has therefore completely abolished signalling suggesting that it may have induced a severe effect on the structure of domain 4. All other mutant receptors were expressed at equivalent levels to hβcFIΔ in the CTL-EN\hIL3Rα cells (data not shown). All receptors with mutations in repeat 2 of hβcFIΔ were capable of mediating growth in the CTL-EN/hIL3Rα cells at low doses of hIL-3 (1ng/ml) as was the hβc mutant (hβcHSV) with an 11 amino acid insertion at position 431. Interestingly, two mutations in repeat 1 (RV-R1 and WS-R1) that abolished the capacity of hβcFIΔ to function without ligand in FDC-P1 cells had no affect on the ability of hβcFIΔ to mediate growth in CTL-EN/hIL3Rα cells in response to hIL-3. We examined these two mutants further to determine whether their capacity to respond to hIL-3 was different to hβcFIΔ. The dose response curves for these hβcFIΔ mutants were identical to unmutated hβcFIΔ. Clearly, the RV-R1 and WS-R1 mutations have not impaired the ability of hβcFIΔ to respond to hIL-3, however they have abolished the factor independence in FDC-P1 cells completely.

Discussion

The shared signalling subunit for IL-3, GM-CSF and IL-5 (hβc) is activated by a small duplicated sequence in domain 4 (D'Andrea *et al* , 1994). To examine the requirements for cytokine receptor activation, mutations were introduced into this segment of hβc and into each copy of the repeated segment in the activated mutant (hβcFIΔ). Altered receptors were analysed for constitutive activity (in FDC-P1 cells) and for their ability to respond to hIL-3 (in CTL-EN cells expressing hIL3Rα). Tables 2 and 3 summarise the effect of each mutation on expression and its activity in these two assays.

Duplicated sequences compensate for the presence of debilitating mutations. As summarised in table 2, none of the introduced mutations abolished cell-surface expression of hβcFIΔ. By monitoring receptor surface expression with hβc monoclonal antibodies we were able to demonstrate that mutations that altered the RVRVR (RV-R1 and RV-R2 mutants) and WSEWS (WS-R1 and WS-R2 mutants) sequences in either of the repeats did not affect expression of hβcFIΔ. This was in contrast to the same mutations in hβc which completely abolished surface expression using our most sensitive detection protocol. Our study is consistent with mutation in these motifs having a deleterious affect on cytokine receptor folding and transport and suggests that receptor folding can be restored in the presence of an additional, unaltered copy of these motifs as in hβcFIΔ. The WSXWS motif has been altered by mutation in several cytokine receptors with varied results (Miyazaki *et al* , 1991; Chiba *et al* 1992; Quelle *et al*1992; Rosakis-Adcock and Kelly, 1992; Yoshimura *et al* , 1992), however, in the most comprehensive analysis, a saturation mutagenesis of the WSXWS motif of mEpoR was performed and the tryptophan and serine residues were shown to be largely intolerant to mutation. Mutation of these residues led to accumulation of mutant receptor in the endoplasmic reticulum (Hilton *et al* , 1995).

In the prolactin receptor the tryptophan residues of the WSXWS motif are in a coplanar arrangement, forming a hydrophobic scaffold, through interactions with methylene groups from side chains of neighbouring polar residues, including arginines from the adjacent conserved basic sequence corresponding to β-strand F' (Somers *et al* , 1994). This structure appears to be conserved throughout the cytokine receptor family. It is not surprising that the alterations in the WSEWS and RVRVR sequences of hβc have similar effects on expression given this predicted intimate interaction. Mutations of key elements of this structure appear to lead to incorrect receptor folding and intracellular accumulation. We propose that in hβcFIΔ the RVRVR and WSEWS sequences in repeat 2 are excluded from domain 4. This is consistent with the receptor polypeptide folding from the N-terminus as it is synthesised, with repeat 1 being incorporated into a relatively normal domain 4 structure. In the presence of the mutations in the WSEWS or RVRVR sequences of repeat 1 the receptor could adopt an alternative conformation in which repeat 2 residues substitute in the domain 4 structure allowing efficient receptor folding and transport . The results of functional studies suggest that these alternative conformations are not equivalent. These experiments are discussed further below.

Effect of mutations on ligand-independent activity in FDC-P1 cells. Several of the mutations that were introduced into the repeated segments were informative in that they differentially affected the ability of the receptor to mediate factor-independence

depending on whether they were introduced into repeat 1 or repeat 2 (see table 2). None of the amino acid substitutions or deletions that we introduced into repeat 2 abolished ligand-independence in FDC-P1 cells. This suggested that activation does not require duplication of specific sequences. As a further test of this we replaced repeat 2 with a completely unrelated sequence, inserted after residue A431 of hβc. Expression of the resultant receptor was equivalent to that of hβcFIΔ on the infected population, and factor-independent FDC-P1 cells were generated after removal of cytokine, albeit at reduced frequency to that observed for hβcFIΔ.

In contrast to the mutations introduced into repeat 2, all of the mutations that were introduced into repeat 1 (apart from Y-R1) abolished the ability of hβcFIΔ to confer factor-independence in FDC-P1 cells. In the presence of the RV-R1 and WS-R1 mutations the altered sequences are presumably replaced by sequences in repeat 2 thus restoring receptor folding and allowing surface expression. However, the resultant receptor is no longer constitutively active in FDC-P1 cells. Thus, it appears that in the presence of mutations in repeat 1 hβcFIΔ takes up a conformation that, although expressed (and functional in CTL-EN/IL3Rα cells in response to hIL-3- see below), is not constitutively activated. This would be consistent with the position of the extruded sequences being a critical factor in activation. Alternative conformations for hβcFIΔ are shown in. Fig 12B shows a possible structure for hβcFIΔ with the sequence of repeat 2 extruded from domain 4. In the alternative conformation (Fig. 12C) most of repeat 2 (excepting the E' β-strand) is included in the domain 4 structure and most of repeat 1 (excluding the E' β-strand) is extruded. This conformation is predicted to form preferentially in the presence of the RV-R1 and WS-R1 mutations. We speculate that the E' strand of repeat 1 is likely to be incorporated into a quasi-normal domain 4 structure in the presence of these mutations for two reasons. Firstly, this part of the repeat is unperturbed and secondly, extrusion N-terminal to the E' strand might be predicted to affect ligand recognition or association with ligand binding subunits on the basis of the known receptor structures. This is clearly not the case as these receptors function identically to unaltered hβc in the presence of hIL-3 (see below). A predicted structure for the hβcHSV mutant is also shown (Fig. 12D). The 11 residue insertion is predicted to be excluded at the point of insertion in a similar manner to repeat 2 of hβcFIΔ. Based on these predictions we suggest that the steric effects of the introduced sequences will vary depending on which residues are excluded from the domain 4 structure. Our results are consistent with activation requiring extruded sequences to be positioned as shown for hβcFIΔ or hβcHSV (Figs. 12B and D).

Two alternative pathways for signalling through hβc. hβcFIΔ functions in an IL-3 dependent fashion in CTL-EN cells expressing the hIL3 specific α-subunit, hIL3Rα.

Other hβc activated mutants (I374N, V449E), while able to mediate ligand-dependent activity also lack constitutive activity in this cell line (Jenkins *et al*, 1995). We used these cells to assay the ability of mutant receptors to interact with ligand in the presence of a specific ligand-binding subunit. All altered forms of hβcFIΔ were expressed at the cell surface in these cells and only the C-terminal deletion of repeat 1 (Δ1-R1) failed to confer an IL-3 response. Hence of three hβcFIΔ mutants that were incapable of conferring ligand-independence in FDC-P1 cells (RV-R1, WS-R1 and Δ1-R1), two with double amino acid substitutions (RV-R1 and WS-R1) retained their ability to signal in response to growth factor in CTL-EN/IL3Rα cells while the deletion in repeat 1 (D1-R1) abolished both functions that we have assayed (see table 2). Dose responses to hIL-3 for the receptors containing the RV-R1 and WS-R1 mutations were not significantly different from that for hβcFIΔ indicating that the binding and response to hIL-3 was not impaired by these mutations. This implies that the alternative conformation of hβcFIΔ that is predicted to form in the presence of these mutations retains the ability to interact with the IL-3 specific alpha subunit in the presence of IL-3 and initiate a response via this pathway.

The fact that it is possible, by introducing amino acid substitutions, to restore wild type function of hβcFIΔ (with respect to IL-3 inducibility), demonstrates that activation has occurred independently of the mechanism for IL-3 stimulation. This is consistent with alternative mechanisms triggering the proliferative responses in the two cell types (FDC-P1 and CTL-EN/hIL3Rα). We have suggested previously that the two classes of hβc activating mutants (transmembrane vs extracellular mutation) may be mimicking alternative modes of ligand-dependent signalling (Jenkins *et al* , 1995). This hypothesis is based on the differential activity of these two classes of mutation in various cell lines. Studies using the FDC-P1, CTL-EN and BaF-B03 haemopoietic cell lines has shown the activity of extracellular activating mutants to be restricted to the murine myeloid cell line FDC-P1 (Jenkins *et al* , 1995) (B. J. Jenkins and T. J. Gonda, submitted). In contrast, another mutant hβcV449E with a substitution in the transmembrane domain can confer factor-independence in both FDC-P1 and BaF-B03 cells. While the V449E mutation possibly activates by inducing an association of transmembrane domains and consequent intracellular oligomerisation, the difference in cellular specificity suggests that the extracellular mutants require a cofactor present in FDC-P1 cells. Thus, the extracellular class of activating mutations appear to be mimicking a normal complex containing an undefined membrane-spanning subunit (ie. αβγ or βγ complexes) while the V449E mutant is more likely to be mimicking a complex containing a ββ dimer.

Model for activation of hβcFIΔ: perturbation of an inhibitory interaction. The lack of sequence constraint observed within repeat 2 of hβcFIΔ and the observation that an unrelated sequence inserted at the same position also leads to activation are consistent with both insertions perturbing an inhibitory interaction that maintains hβc in the inactive state. We have previously suggested that one mechanism of hβc activation requires unmasking of an epitope in domain 4 that mediates an intermolecular interaction (with a proposed γ-subunit present in FDC-P1 cells and absent in lymphoid cell lines) and generates a proliferative signal. This was based on the observation that hβc can be activated by extracellular truncations that remove the first three N-terminal fibronectin-like domains suggesting that domain 3 may be involved in masking an interactive site in domain 4 (D'Andrea *et al*, 1996). We suggest that mutations in hβcFIΔ (such as RV-R1 and WS-R1) can selectively prevent its association with the putative γ-subunit while not affecting its ability to associate with IL3Rα and mediate a response to ligand. While these mutations restore inhibition and wild type function, a more severe effect is observed with the Δ1-R1 mutant which abolishes wild type receptor function.

Implications for activation of cytokine receptors. We have suggested previously that hβc is maintained in an inactive state via an inhibitory mechanism that can be affected by mutation (D'Andrea *et al*, 1996). From this work with truncated hβc mutants we suggested that domain 3 of hβc may have an involvement in this inhibition. An activated form of MPL (the thrombopoietin receptor), that is a truncated receptor fusion (v-MPL) (Souyri *et al*, 1990) has been found suggesting that similar regulatory mechanisms may operate for other cytokine receptors. Our favoured model invokes direct masking of an interactive site in domain 4 by other receptor determinants including some from domain 3. Another possible mechanism for inhibition is association with a membrane spanning phosphatase. A variety of protein tyrosine phosphatases (PTPases) have been described (Fischer *et al*, 1991) and one of these, termed haematopoietic cell phosphatase (HCP), is a cytoplasmic protein containing 2 amino-terminal SH2 domains, the first of which facilitates association with hβc (Yi *et al*, 1993), *c-kit* (Yi and Ihle, 1993), and Epo-R (Yi *et al*, 1995; Klingmuller *et al*, 1995). As yet no membrane-spanning phosphatases have been described in association with hβc.

In hβc and in many other cytokine receptors, binding determinants are located in the membrane-proximal domain of the CRM (DeVos, 1992; Woodcock *et al* , 1994; Bass *et al*, 1991; Wand *et al*, 1992; Imler *et al*, 1992). In hβc several activating mutations cluster in this domain (Jenkins *et al* , 1995; D'Andrea *et al*, 1994) (B. J. Jenkins and T.

J. Gonda, submitted) consistent with it having a regulatory role in receptor activation. Given this and the conserved structure that has been demonstrated for this family of receptors it is reasonable to predict that common mechanisms of activation will exist in this receptor family. Certainly there are common themes emerging with respect to the intracellular machinery required for signalling (Ihle1, Ihle2). Our work suggests it will be feasible to use peptides or other small molecules, interacting with domain 4 of hβc, in a specific position, to activate the receptor by preventing an inhibitory interaction. A prediction from these studies is that antibodies or small molecules with an affinity for this domain of cytokine receptors may yield receptor agonists. These are proposed to provide the basis of novel therapeutics based on small molecule cytokine receptor binders.

It will be understood that this work has been conducted in mice cells and that the interaction in a purely human cell system may be subtly different. The above results may be influenced by a murine specific factor.

EXAMPLE 5

Generation of antibody agonists to the β3 or β4 domains

Monoclonal antibodies can be generated by immunising with fragments of the β3 or β4 domain and screen for those that bind the β3 or β4 domains. Once a specific monoclonal antibody has been identified and shown to provide for mAB-dependent activation of the β_c, smaller fragments may be generated; e.g. $F(ab)_2$, Fab and ultimately Fv. By molecular biology techniques a small Fv fragment can be constructed (Hv-Lv). This would be a small molecule agonist.

EXAMPLE 6

Generation of agonists to the β3 or β4 domains- oligonucleotides

A library of randomly synthesized oligonuclotides can be passed through a solid matrix to which is bonded a fragment of the β3 or β4 (Bock *et al.*, 1992 - which reference is incorporated herein). Following washing the strongly binding oligonucleotides remain and can then be eluted under different conditions (salt, ph etc). The sequence can be determined by PCR and tested for activation on a cell system.

EXAMPLE 7

Generation of peptide agonists to the β3 or β4 domains

A scramble of randomly generated short peptides or phage display library may be synthesised and passed through a column as described in example 6. Those that bind can then be tested for factor independent activation on a real cell system.

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CLAIMS

1. A therapeutic agent said agent being an agonist to a receptor of the cytokine receptor family having an extracellular receptor module (CRM) composed of two discrete folding domains (CRD), a first of the two CRDs being a switching CRD and a second of the CRDs being an inhibiting CRD said CRM being in an inactive state unless activated by ligand, said agonist property being a result of a change in the switching CRD from its inactive state to an active state leading to a signal being produced to effect said agonist property.

2. A therapeutic agent as in claim 1 wherein the agent interferes with interaction of the inhibiting CRD with the switching CRD.

3. An agent as in claim 1 wherein the agent interference with the interaction between sheet 1 and sheet 2 of the switching CRD.

4. An agent as in claim 3 wherein the agent specifically binds with a portion of the switching CRD including the B' strand of switching CRD.

5. An agent as in claim 3 wherein the agent specifically binds with a portion of the switching CRD including the C' strand of the switching CRD.

6. An agent as in claim 3 wherein the agent interferes with the interaction between any one or more of isoleucine 374, leucine 356, tryptophan 358 or tyrosine 376 and an apposed amino acid in hβc or analogous amino acids in another member of the cytokine receptor family.

7. A therapeutic agent as in claim 1 wherein the agent is a peptide with an amino acid sequence substantially identical to an amino acid sequence present in the inhibiting CRD domain or the switching CRD domain of a respective haemopoietic growth factor.

8. An agent as in claim 7 wherein the agent has an amino acid sequence substantially present in the B' strand of the switching CRD.

9. An agent as in claim 7 wherein the agent has an amino acid sequence substantially present in the C' strand of the switching CRD.

10. An agent as in claim 7 wherein the agent has an amino acid sequence including a sequence selected from a group of fragments including the membrane proximal WSXWS motif and the YXXRVRVR motif located in the switching CRD.

11. An agent as in claim 7 wherein the agent includes the WSXWS motif located in the switching CRD.

12. An agent as in claim 7 wherein the agent includes the RVRVR motif located in the switching CRD.

13. An agent as in claim 7 wherein the agent has an amino acid sequence present in either the B or the C strand of a switching CRD and includes any one or more of isoleucine 374, leucine 356 or tryptophan 358 in hβc or analogous amino acids in other CRM.

14. An agent as in claim 1 wherein the member of the cytokine receptor family is selected from a group acting as a receptor of the any one or more of the following cytokines GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-13, IL-14, IL-15, granulocyte colony stimulating factor (G-CSF), erythropoietin (EPO), thrombopoietin (TPO) and leptin.

15. An agent as in claim 1 wherein the haemopoietic growth factor is any one or more of GM-CSF, IL-5 and IL-3.

16. An agent as in claim 1 wherein the agent is selected from any one of a number of classes of compounds comprising antibodies, fragments of antibodies, peptides, oligosaccharides, oligonucleotides, or other organic or inorganic compounds.

17. An agent as in claim 1 capable of binding one or more of those parts of the inhibiting CRD or switching CRD that are conserved within the cytokine receptor family.

18. An agent as in claim 1 capable of binding a region of the switching CRD including at least a portion of the B' strand of switching CRD.

19. An agent as in claim 1 capable of binding a region of the switching CRD including at least a portion of the C' strand of switching CRD.

20. An agent as in claim 1 capable of binding a region of the switching CRD including the WSXWS motif or the YXXRVRVR motif.

21. An agent as in claim 1 capable of binding a region of the switching CRD including the WSXWS motif.

22. An agent as in claim 1 capable of binding a region of the switching CRD including the RVRVR motif.

23. An agent as in claim 1 capable of binding Isoleucine 374 in hβc or analogous amino acid residue in another member of the cytokine receptor family.

24. An agent as in claim 1 capable of binding a region of the switching CRD including isoleucine 374 or leucine 356 or tryptophan 358 in hβc or analogous amino acid residue in another CRM member of the cytokine receptor family.

25. An agent as in claim 1 capable of binding a region of the switching CRD including amino acid leucine 356 or tryptohan 358 in hβc or analogous amino acid residues in another member of the cytokine receptor family.

26. An agent as in claim 1 capable of binding portions of the inhibiting CRD or switching CRD at an interface between inhibiting CRD and the switching CRD.

27. A method for isolating a therapeutic agent said agent being a receptor of the cytokine receptor family having an extracellular receptor module (CRM) composed of two discrete folding domains (RD), a first of the two CRDs being a switching CRD and a second of the CRDs being an inhibiting CRD said CRM molecule being in an inactive state unless activated by ligand, said agonist property being a result of a change in the switching CRD from its inactive state to an active state leading to a signal being produced to effect said agonist property

said method including the steps of contacting candidate agents with fragments of the inhibiting CRD or the switching CRD fragments, assaying candidate agents for their capacity to bind said inhibiting CRD or switching CRD fragments and testing for agonist properties.

28. The method as in claim 27 including fixing the fragments to a substrate, contacting the fragments with the candidate agents, washing away unbound material not bound to the substrate including unbound candidate agents, separating the bound

candidate agents from the fragments or said substrate, identifying the bound candidate agents, testing the agents for agonist properties.

29. An method as in claim 27 wherein the member of the cytokine receptor family is selected from a group acting as a receptor of the any one or more of the following cytokines GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-13, IL-14, IL-15, granulocyte colony stimulating factor (G-CSF), erythropoietin (EPO), thrombopoietin (TPO) and leptin.

30. A method as in claim 27 wherein the haemopoietic growth factor is any one or more of GM-CSF, IL-5 and IL-3.

31. A method as in claim 27 wherein the candidate agent is selected form of any one of a number of classes of compounds comprising antibodies, fragments of antibodies, peptides, oligosaccharides, oligonucleotides, or other organic or inorganic compounds.

32. A method as in claim 27 wherein the candidate agent is a peptide with an amino acid sequence substantially identical to an amino acid sequence present in the inhibiting CRD or the switching CRD of a respective member of the cytokine receptor family.

33. A method as in claim 32 wherein the candidate agent has an amino acid sequence substantially present in the B strand of the switching CRD.

34. A method as in claim 32 wherein the candidate agent has an amino acid sequence substantially present in the C strand of the switching CRD.

35. A method as in claim 32 wherein the candidate agent has an amino acid sequence including a sequence selected from a group of fragments including the membrane proximal WSXWS motif and the YXXRVRVR motif located in the switching CRD.

36. A method as in claim 32 wherein the candidate agent includes the WSXWS motif located in the switching CRD.

37. A method as in claim 32 wherein the candidate agent includes the RVRVR motif located in the switching CRD.

38. A method as in claim 32 wherein the candidate agent has an amino acid sequence present in either the B or the C strand of the switching CRD and includes any

one or more of isoleucine 374, leucine 356 or tryptophan 358 in hβc or analogous amino acids in another member of the cytokine receptor family.

39. A method as in claim 27 wherein the fragments comprise one or more of those parts of the inhibiting CRD or switching CRD that are conserved within the family of haemopoietic growth factor receptors.

40. A method as in claim 27 wherein the fragments comprise a region of the switching CRD including at least a portion of the B strand of switching CRD.

41. A method as in claim 27 wherein the fragments comprise a region of the switching CRD including at least a portion of the C strand of switching CRD.

42. A method as in claim 27 wherein the fragments comprise a region of the switching CRD including the WSXWS motif or the YXXRVRVR motif.

43. A method as in claim 27 wherein the fragments comprise a region of the switching CRD including the WSXWS motif.

44. A method as in claim 27 wherein the fragments comprise a region of the switching CRD including the RVRVR motif.

45. A method as in claim 27 wherein the fragments comprise isoleucine 374 in hβc or analogous amino acid residue in another member of the cytokine receptor family.

46. A method as in claim 27 wherein the fragments comprise a region of the switching CRD including isoleucine 374 or leucine 356 or tryptophan 358 in hβc or analogous amino acid residue in another member of the cytokine receptor family.

47. A method as in claim 27 wherein the fragments comprise a region of the switching CRD including amino acid leucine 356 or tryptohan 358 in hβc or analogous amino acid residues in another member of the cytokine receptor family.

48. A method as in claim 27 wherein the fragments are peptides with amino acid sequences close to the amino acid sequences of amino acids at an interface between the inhibiting CRD and the switching CRD.

49. A method as in claim 32 wherein the fragments include any sugar moieties that is associated with the inhibiting CRD or switching CRD.

50. A method of treating a condition in a human or an animal, by administering an agonist of a member of the cytokine receptor family in a pharmaceutically acceptable form in a suitable carrier, and in a therapeutically effective dose, said agonist as in any one of claims 1 to 26.

Table 1. Summary of activating mutations in βc.

MUTANT	RECEPTOR/ DOMAIN AFFECTED	COMMENT
V449E	hβc Transmembrane domain	Likely to mediate homodimerisation
FIΔ	hβc Extracellular domain 4	37 aa duplication
HSV	hβc Extracellular domain 4	Insertion of 11 amino acids
Truncation mutants ΔQP, ΔH,	hβc Extracellular domain 4	Truncations removing domains 1, 2 and 3
I374N Also I374D, I374Q, I374F	hβc Extracellular domain 4	Point mutation in strand C'
W358N, L356N	hβc Extracellular domain 4	Point mutations in strand B'
rearranged mβc gene	mβc Extracellular domain	May induce disulfide-linked dimerization

Table 2. Targeted mutagenesis of the repeated segments of hβcFIΔ

MUTATION	SEQUENCE	REPEAT SEGMENT	SURFACE EXPRESSION[a]	FACTOR INDEPENDENCE[b]	hIL-3 DEPENDENT GROWTH (CTL-EN/IL3Ra)
Y-R1	YWARVRVR	1	+	+	+
	Ø				
	GWARVRVR				
Y-R2		2	+	+	+
RV-R1	YWARVRVR	1	+	-	+
	Ø				
	YWARERVA				
RV-R2		2	+	+	+
WS-R1	WSEWSÆSSESS	1	+	-	+
WS-R2		2	+	+	+
Δ1-R1[c]		1	+	-	-
Δ1-R2		2	+	+	+
Δ2-R2		2	+	+	+

a. Surface expression was determined by flow cytometric analysis following immunostaining with an hbc monoclonal antibody.

b. All mutant hβc cDNAs were cloned into retroviral vectors and factor independence determined after infection of FDC-P1 cells.

c. See Figure 10 for details of deletions.

Table 3. Alteration of conserved motifs in hβc.

MUTATION	SEQUENCE	SURFACE EXPRESSION[a]	FACTOR INDEPENDENCE[b]	hIL-3 DEPENDENT GROWTH (CTL-EN/IL3Ra)
Y-wt	YWARVRVR Ø GWARVRVR	+	-	+
RV-wt	YWARVRVR Ø YWARERVA	-	-	-
WS-wt	WSEWSÆSSESS	-	-	-

a. Surface expression was determined by flow cytometric analysis following immunostaining with an hβc monoclonal antibody.
b. All mutant hβc cDNAs were cloned into retroviral vectors and factor independence determined after infection of FDC-P1 cells.

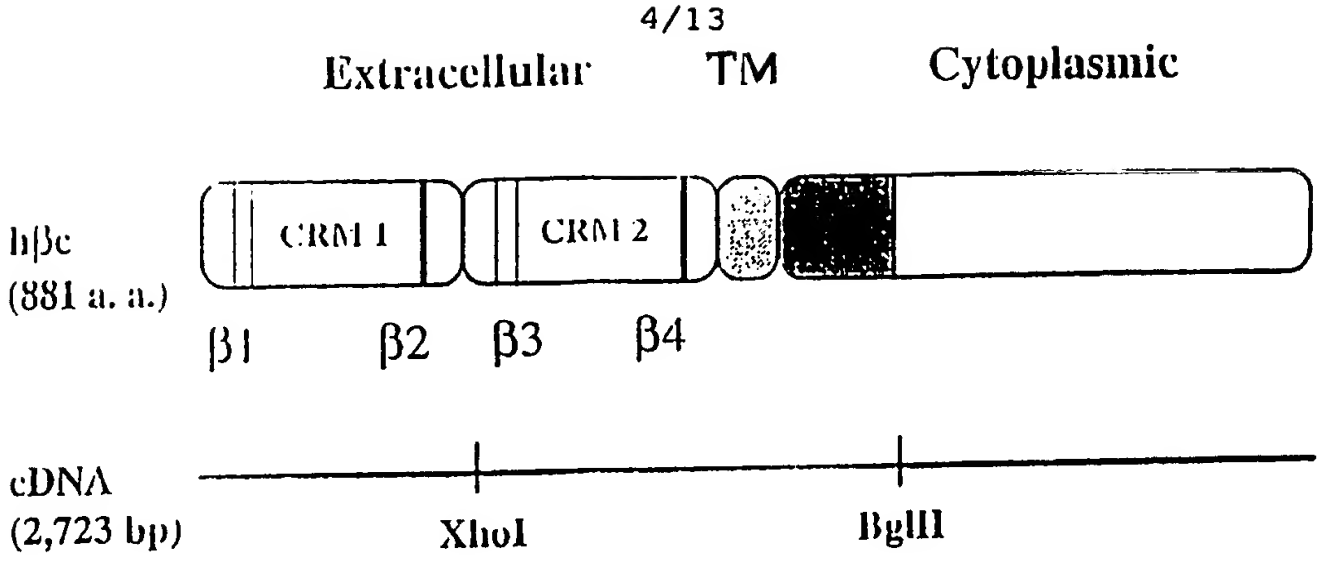

FIGURE 1

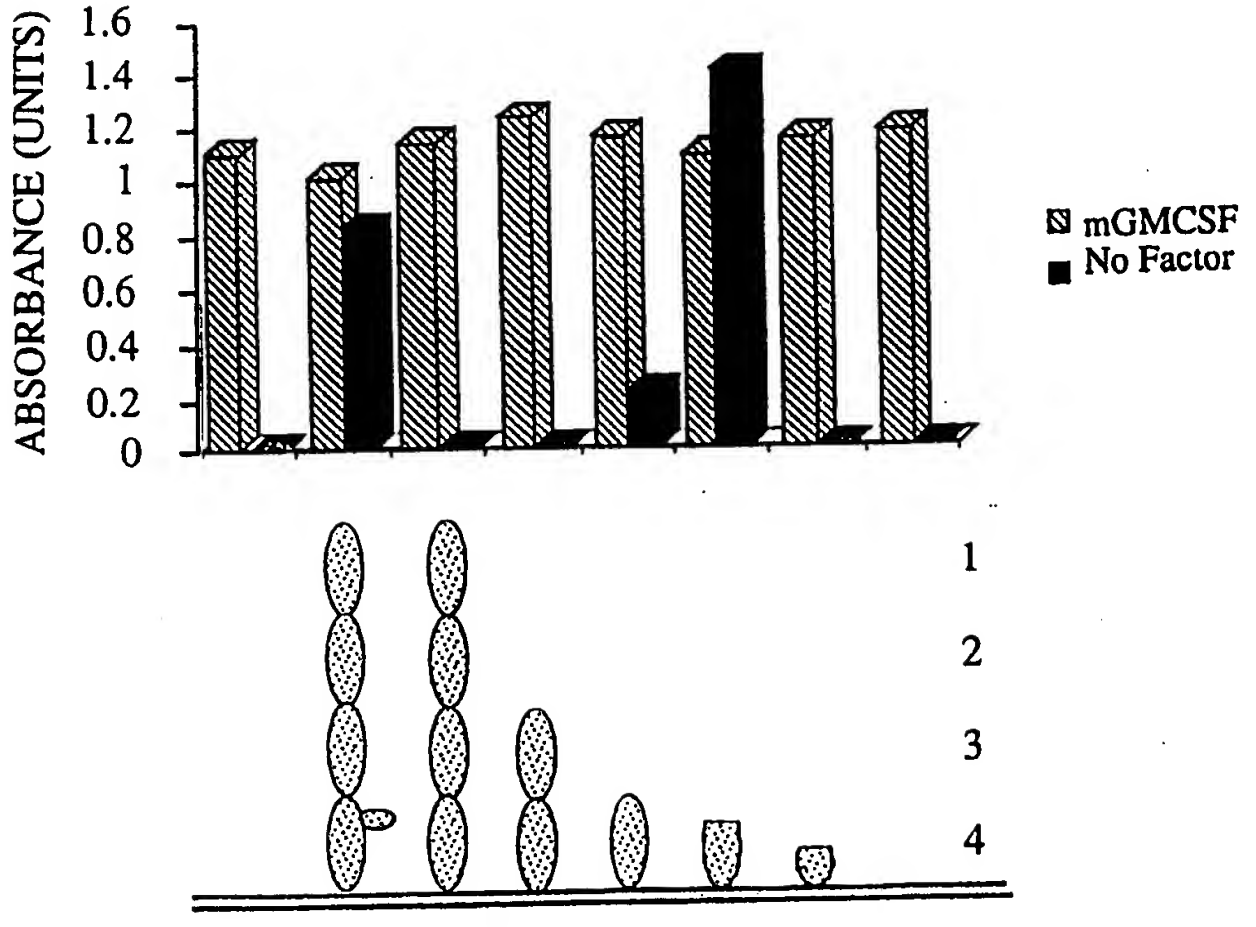

FIGURE 3

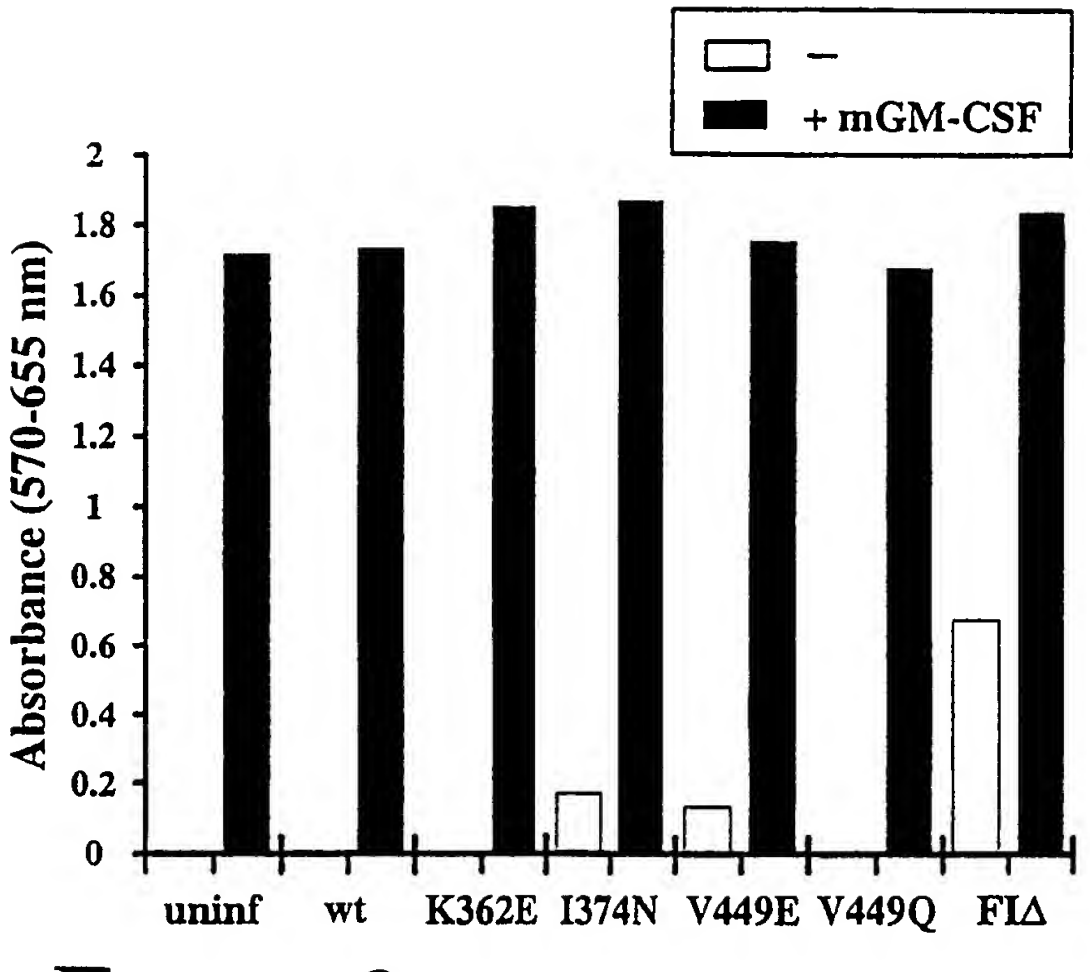

FIGURE 2A

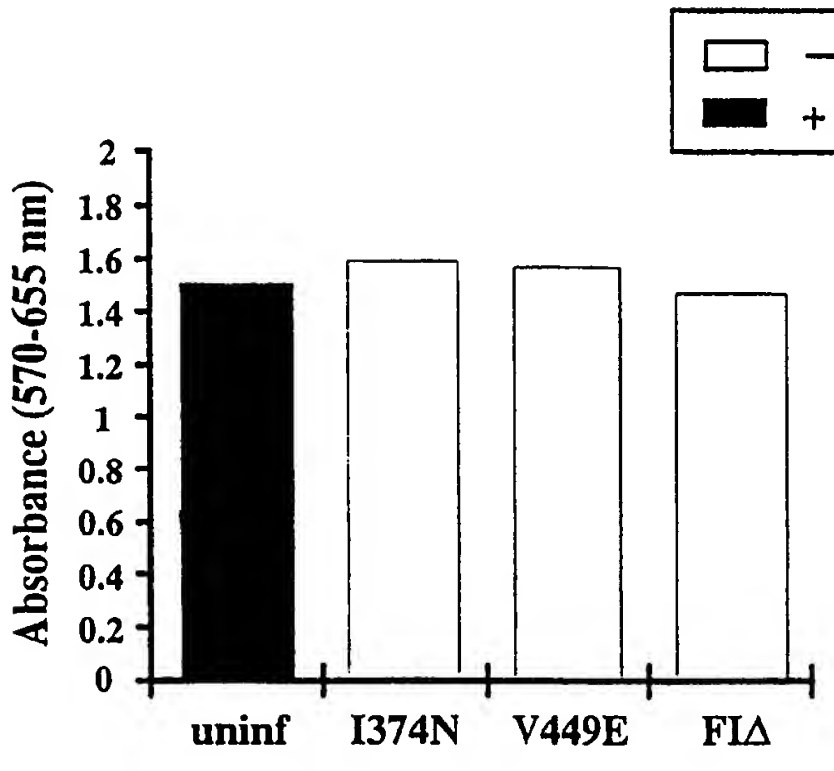

FIGURE 2B

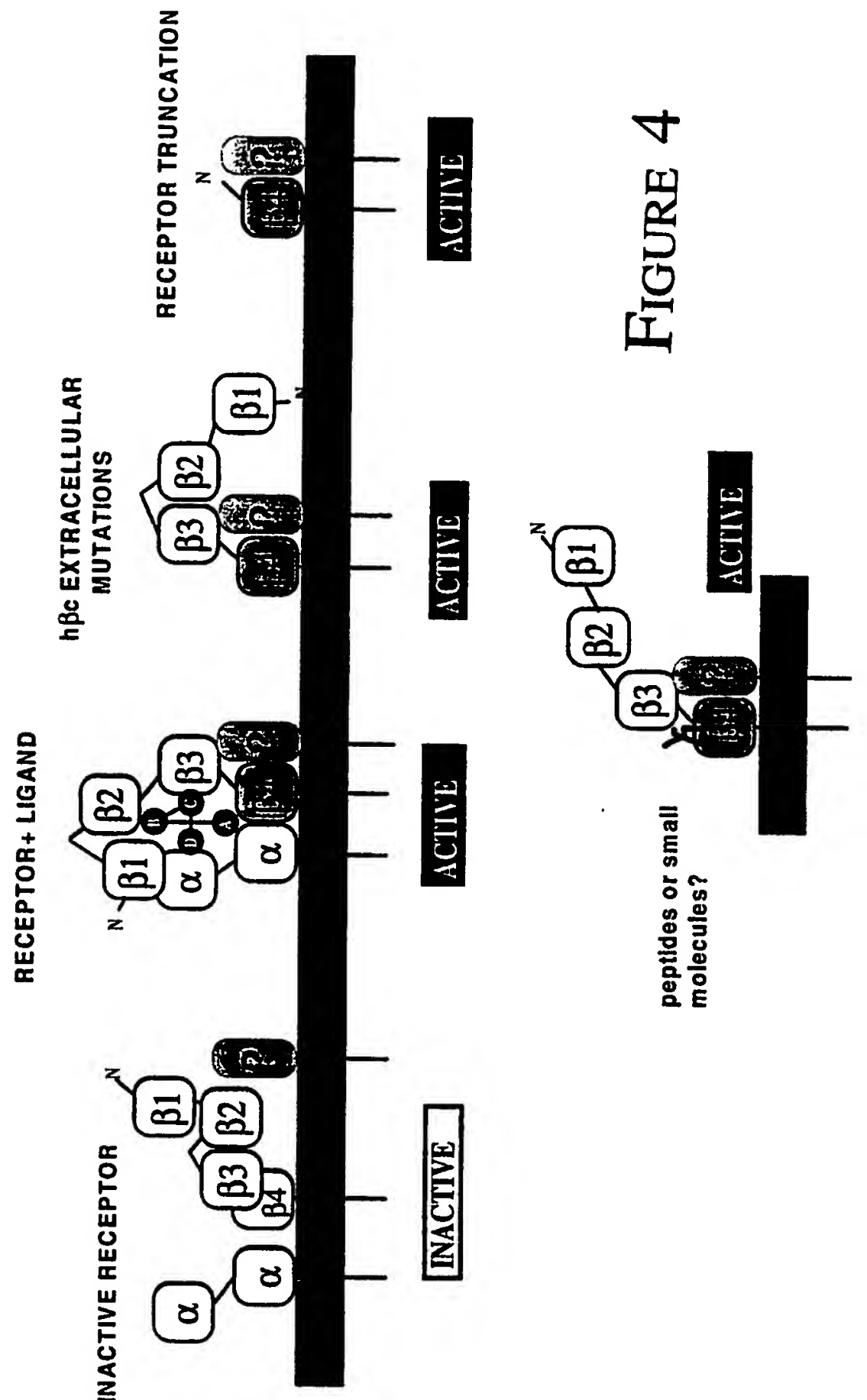
Activation of βc
hβc EXTRACELLULAR MUTATIONS
RECEPTOR TRUNCATION
ACTIVE
ACTIVE
RECEPTOR+ LIGAND
ACTIVE
INACTIVE RECEPTOR
INACTIVE
peptides or small molecules?
ACTIVE

FIGURE 4

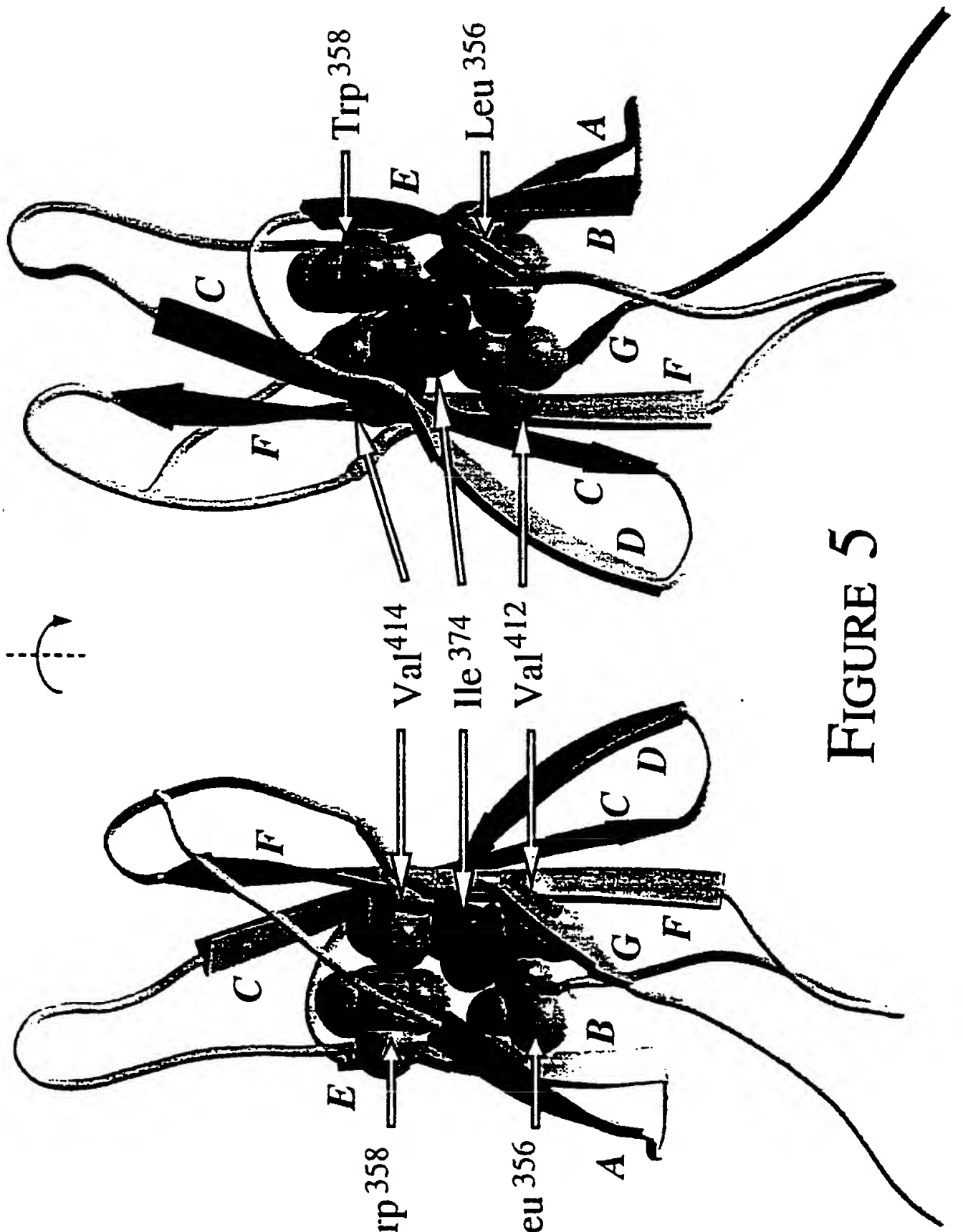

FIGURE 5

```
              356 358                       374                               412 414
βc    D S Y S L R W E T M K M R - - - Y E H I D H T F E I Q Y R K ... Y W A R V R V R T S R
GHR   A D I Q V R W E A P R N A D I Q K G W M V L E Y E L Q Y K E ... K E Y E V R V R S K Q
c-Mpl G H L E L E W Q H P S S W - - - - A A Q E T C Y Q L R Y T G ... Y R L Q L R A R L N G
gp130 N E A V L E W D Q L P V D V Q - - N G F I R N Y T I F Y R T ... Y M V R M A A Y T D E
IL-2β H R C N I S W E I S Q A S - - - - H Y F E R H L E F E A R T ... Y E F Q V R V K P L Q
IL-2γ S Q L E L N W N N R F L - - - - - - N H C L E H L V Q Y R T ... Y T F R V R S R F N P
```

FIGURE 6

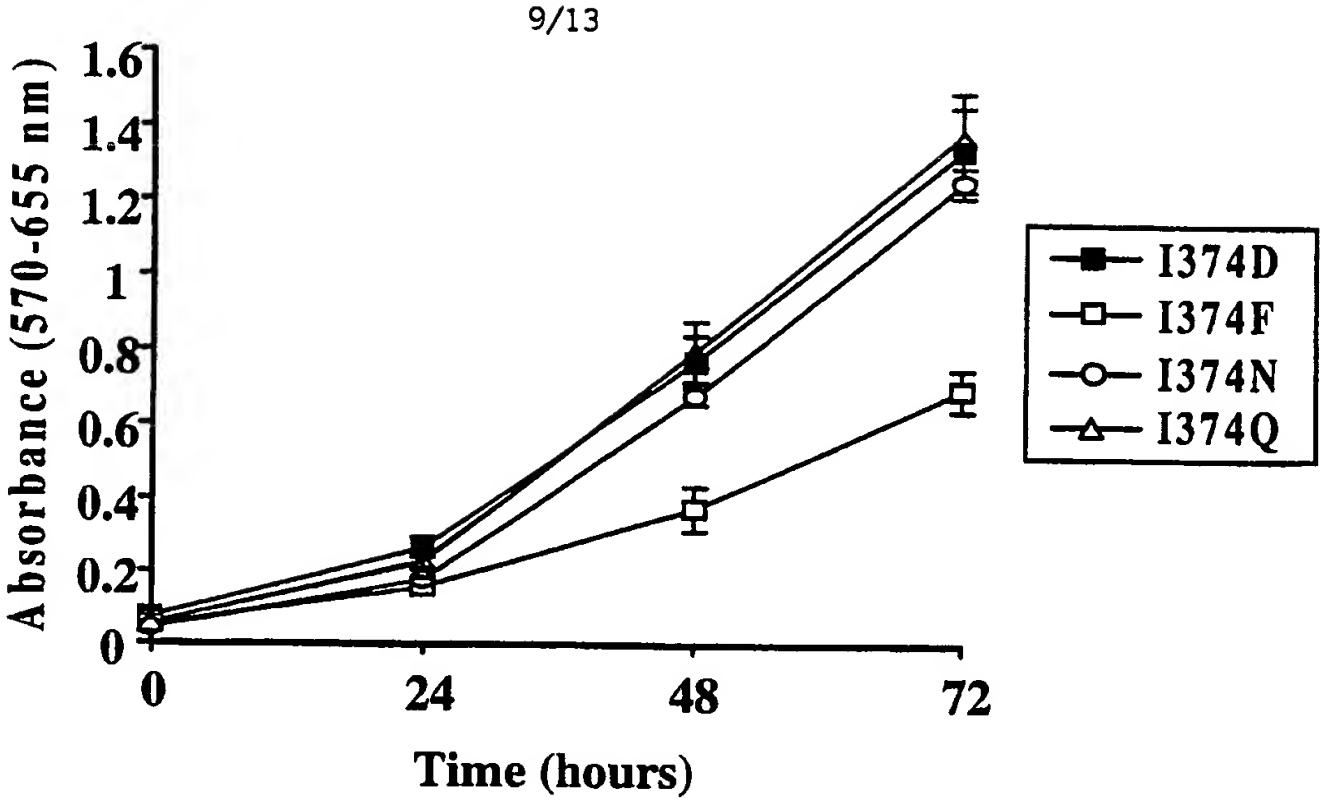

FIGURE 7A

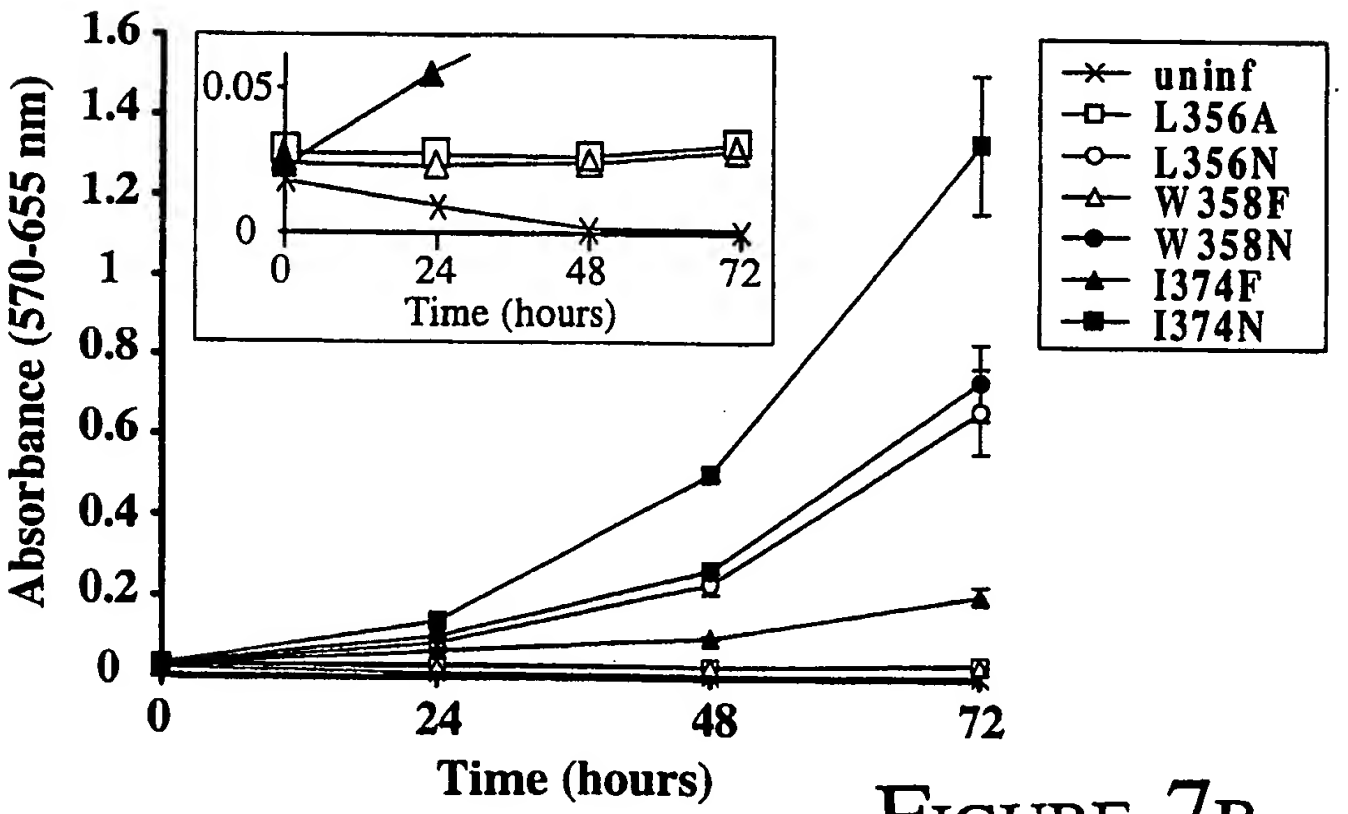

FIGURE 7B

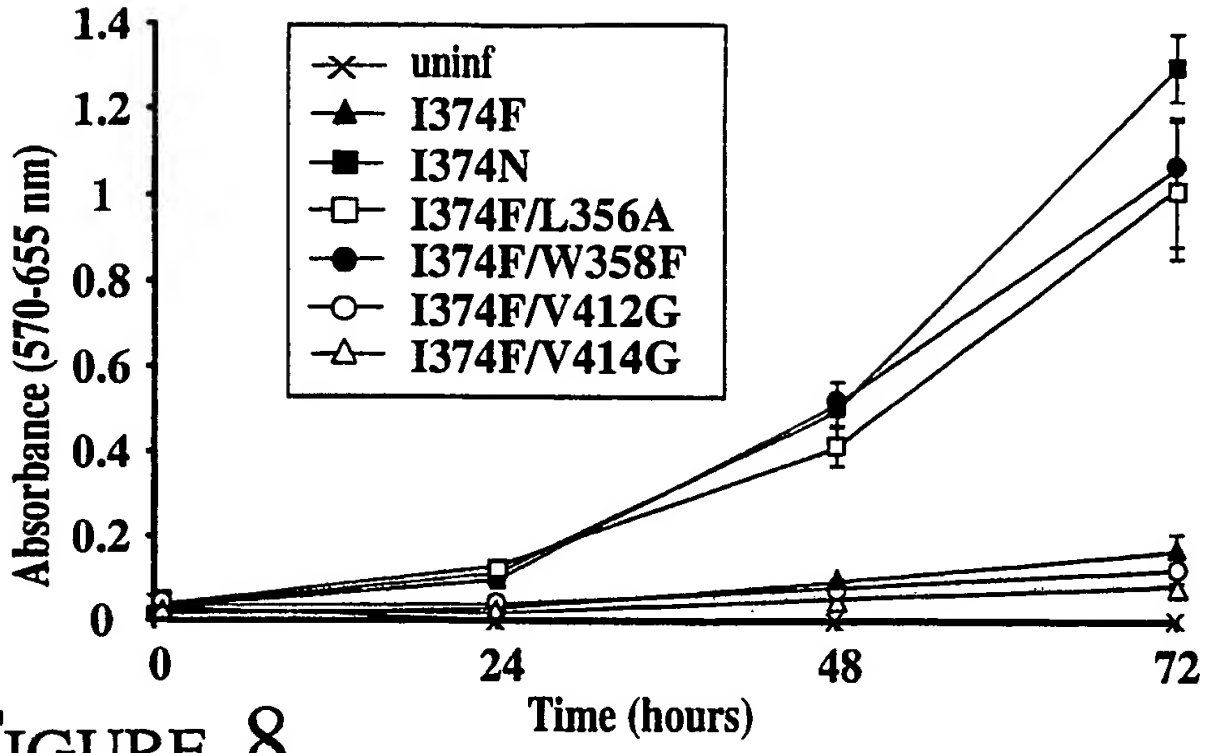

FIGURE 8

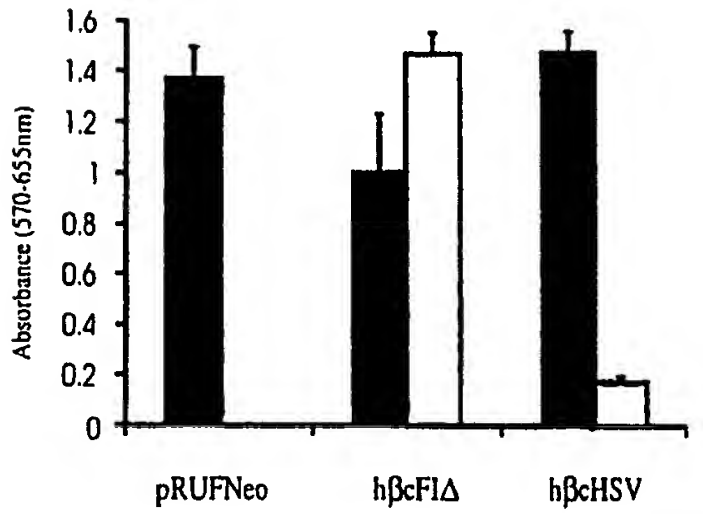

FIGURE 11A

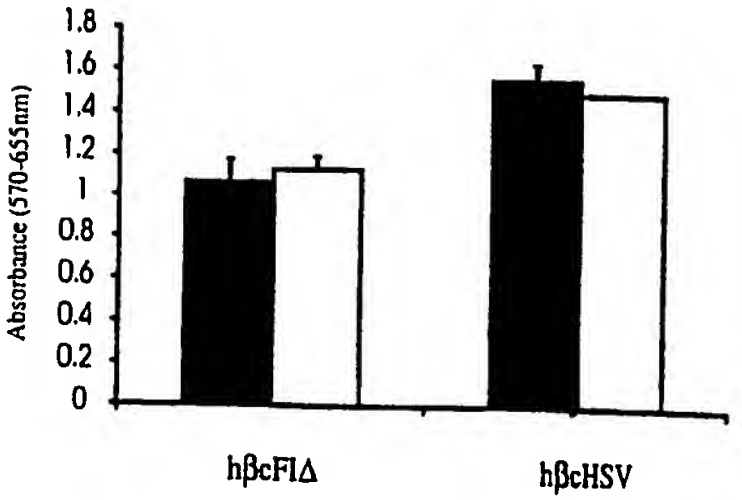

FIGURE 11B

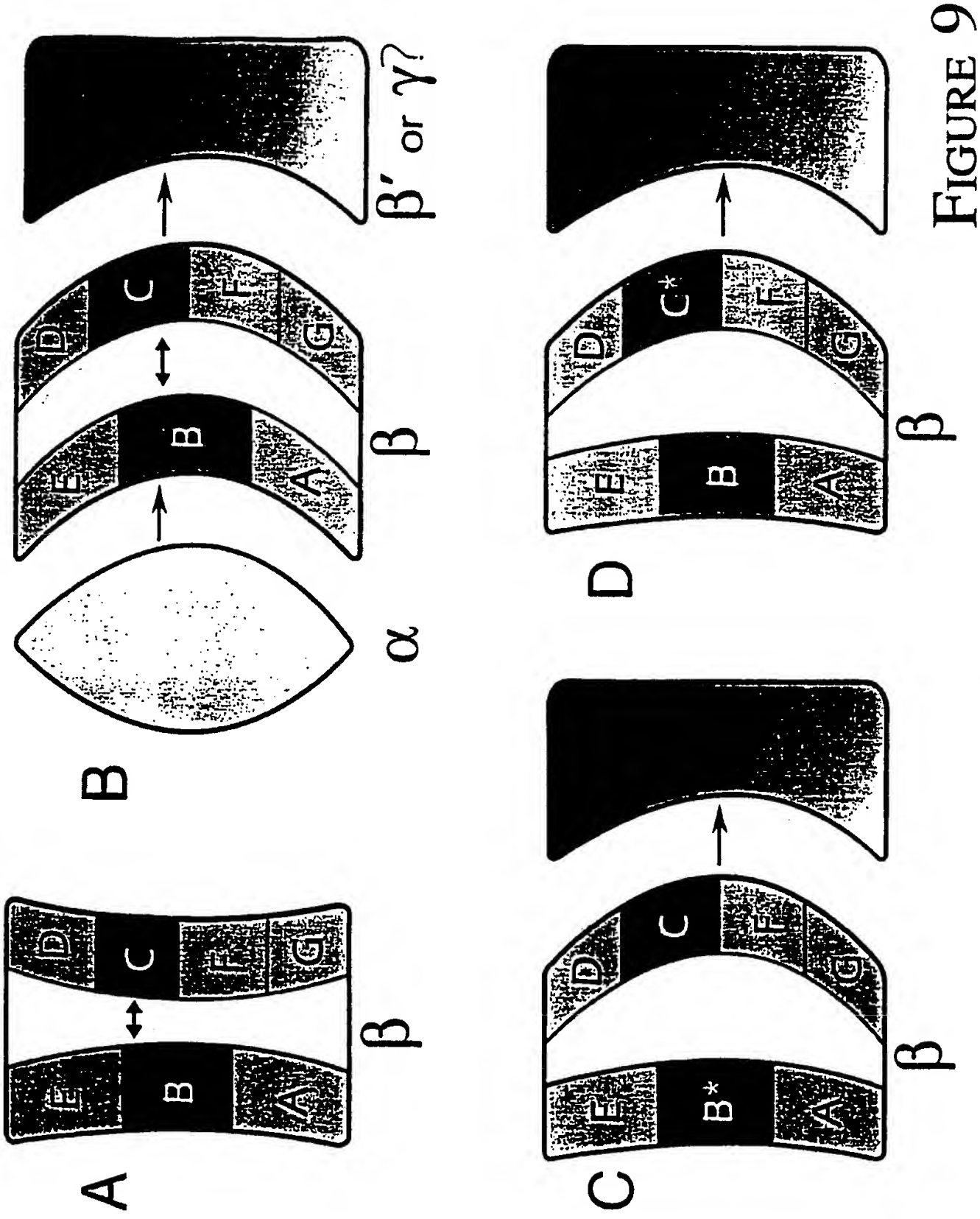

FIGURE 9

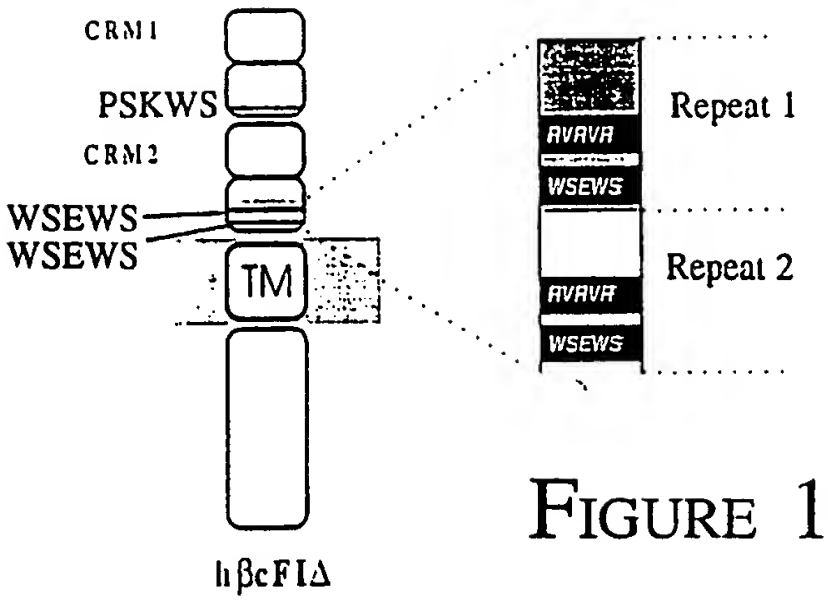

FIGURE 10A

repeat 1

A431

hβcFIΔ LQNAHSMALPALEPSTRYWARVRVRTSRTGYNGIWSEWSEA

R432

HSMALPALEPSTRYWARVRVRTSRTGYNGIWSEWSEARSWDTES VLPMWVL

repeat 2

FIGURE 10B

repeat 2

hβcFIΔ WSEWSEAHSMALPALEPSTRYWARVRVRTSRTGYNGIWSEWSEARSWD

Δ1 WSEWSEA SS RVRTSRTGYNGIWSEWSEARSWD

Δ2 WSEWSEAHSMALPALEPSTRYWARV RSWD

FIGURE 10C

FIGURE 10D

hβcHSV WARVRVRTSRTGYNGIWSEAQSELAPEDPEDRSWDTESVLPMWVLAL

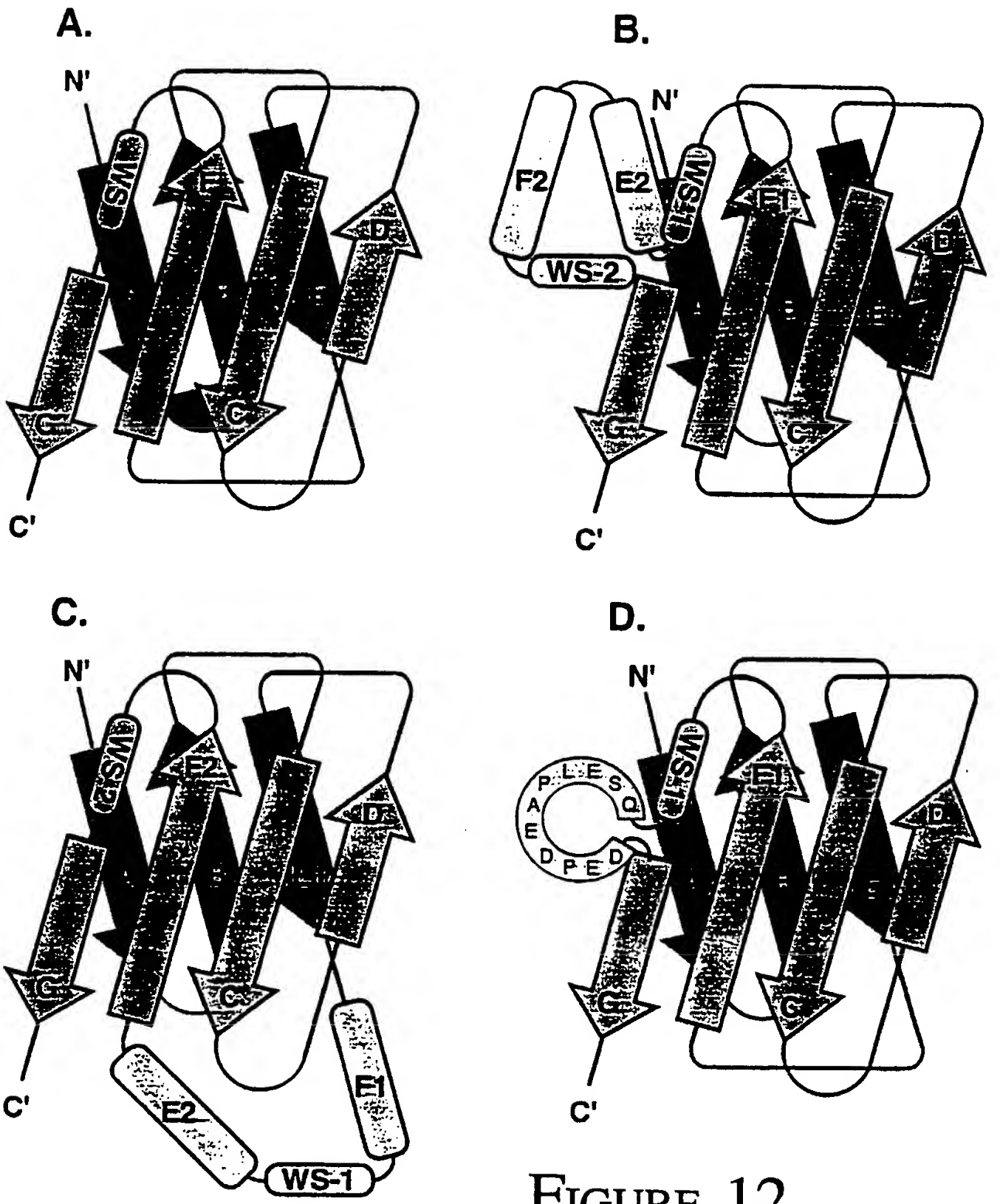
A.
N'
WSM
F
D
G
C
C'
B.
N'
F2
E2
WSM
WS-2
F
D
G
C
C'
C.
N'
WSM
F2
D
G
C
E2
E1
WS-1
C'
D.
N'
WSM
P L E S Q
A
E
D P E D
F
D
G
C
C'

FIGURE 12

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00521

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 15/24, 15/27

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC : As above

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT : IPC As above

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AU,B, 67334/87 (610057) (SCHERING-BIOTECH CORPORATION) 2 June 1987	
A	AU,B, 27827/89 (611750) (SCHERING-BIOTECH CORPORATION) 5 May 1989	
A	AU, A, 73414/94 (MEDVET SCIENCE PTY LTD) 9 February 1995	
A	AU,A, 73996/94 (SCHERING-BIOTECH CORPORATION) 2 February 1995	

☐ Further documents are listed in the continuation of Box C

☒ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 September 1996	9 Oct 1996
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929	Authorized officer **BARRY SPENCER** Telephone No.: (06) 283 2284

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00521

Information on patent family members

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU	67334/87	US	5041381	AT	99332	AU	611750
		CA	1335653	CN	1032816	DE	3886760
		DK	169627	EP	314402	EP	375743
		ES	2061736	HK	1059/96	IE	62491
		IL	88145	KR	9308112	NZ	226692
		PT	88847	ZA	8807987	WO	8903846
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		DE	3650538	DK	3710/87	EP	230107
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		JP	8084591	KR	9309084	KR	9407773
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		NO	960309	PL	312718	WO	950411
		ZA	9405434				
AU	73414/94	CA	2168261	EP	715633	WO	950407

END OF ANNEX